THE CHEMISTRY OF ENZYME ACTIONS

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Press of

GENERAL INTRODUCTION

American Chemical Society Series of Scientific and Technologic Monographs

By arrangement with the Interallied Conference of Pure and Applied Chemistry, which met in London and Brussels in July, 1919, the American Chemical Society was to undertake the production and publication of Scientific and Technologic Monographs on chemical subjects. At the same time it was agreed that the National Research Council, in cooperation with the American Chemical Society and the American Physical Society, should undertake the production and publication of Critical Tables of Chemical and Physical Constants. The American Chemical Society and the National Research Council mutually agreed to care for these two fields of chemical development. The American Chemical Society named as Trustees, to make the necessary arrangements for the publication of the monographs, Charles L. Parsons, Secretary of the American Chemical Society, Washington, D. C.; John E. Teeple, Treasurer of the American Chemical Society, New York City; and Professor Gellert Alleman of Swarthmore College. The Trustees have arranged for the publication of the American Chemical Society series of (a) Scientific and (b) Technologic Monographs by the Chemical Catalog Company of New York City.

The Council, acting through the Committee on National Policy of the American Chemical Society, appointed the editors, named at the close of this introduction, to have charge of securing authors, and of considering critically the manuscripts prepared. The editors of each series will endeavor to select topics which are of current interest and authors who are recognized as authorities in their respective fields. The list of monographs thus far secured appears in the publisher's own announcement elsewhere in this volume.

The development of knowledge in all branches of science, and especially in chemistry, has been so rapid during the last fifty years it is difficult for any individual to keep in touch with the progress in branches of science outside his own specialty. In spite of the facilities for the examination of the literature given by Chemical Abstracts and such compendia as Beilstein's Handbuch der Organischen Chemie, Richter's Lexikon, Ostwald's Lehrbuch der Allgemeinen Chemie, Abegg's and Gmelin-Kraut's Handbuch der Anorganischen Chemie and the English and French Dictionaries of Chemistry, it often takes a great deal of time to coordinate the knowledge available upon a single topic. Consequently when men who have spent years in the study of important subjects are willing to coordinate their knowledge and present it in concise, readable form, they perform a service of the highest value to their fellow chemists.

It was with a clear recognition of the usefulness of reviews of this character that a Committee of the American Chemical Society recommended the publication of the two series of monographs under the auspices of the Society.

Two rather distinct purposes are to be served by these monographs. The first purpose, whose fulfilment will probably render to chemists in general the most important service, is to present the knowledge available upon the chosen topic in a readable form, intelligible to those whose activities may be along a wholly different line. Many chemists fail to realize how closely their investigations may be connected with other work which on the surface appears far afield from their own. These monographs will enable such men to form closer contact with the work of chemists in other lines of research. The second purpose is to promote research in the branch of science covered by the monograph, by furnishing a well digested survey of the progress already made in that field and by pointing out directions in which investigation needs to be extended. To facilitate the attainment of this purpose, it is intended to include extended references to the literature, which will enable anyone interested to follow up the subject in more detail. If the literature is so voluminous that a complete bibliography is impracticable, a critical selection will be made of those papers which are most important.

The publication of these books marks a distinct departure in the policy of the American Chemical Society inasmuch as it is a serious attempt to found an American chemical literature without primary regard to commercial considerations. The success of the venture will depend in large part upon the measure of cooperation which can be secured in the preparation of books dealing adequately with topics of general interest; it is earnestly hoped therefore that every member of the various organizations in the chemical and allied industries will recognize the importance of the enterprise and take sufficient interest to justify it.

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For additional information regarding this series of monographs, see General Introduction, page 3. As the number of copies of any one monograph will be limited, advance orders are solicited.

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AUTHOR'S PREFACE

The relations and theories of chemistry obviously are of paramount importance in enzyme studies. At the same time, enzyme studies have contributed in the past and will unquestionably in the future continue to contribute to a better understanding of certain phases of chemical science. Such will be the case not only in relation to the development of the knowledge of the chemical phenomena underlying living matter, but also, and this statement may be considered somewhat surprising, in connection with a better understanding of the fundamental chemical relations underlying an exact knowledge of chemical reactions. As this last statement may require further explanation, the following considerations in support of it are offered as a possible illustration.

The great importance of the hydrogen ion concentration of the medium in enzyme actions was emphasized first by Sörensen in 1909. Its significance has since been brought out repeatedly. In reactions of inorganic chemistry, such as the changes used in qualitative and quantitative analyses, acidity and alkalinity of solutions are often spoken of, but exact measures of hydrogen ion concentrations used only infrequently. Just as exact measures of hydrogen ion concentrations were accompanied by great advances in the study of enzyme actions, so similar striking advances in the science of analytical chemistry may be expected when exact measures of hydrogen ion concentrations are used generally. Reference may be made to an example of this in an investigation of the study of the conditions affecting the precise determination of zinc as the sulfide (H. T. Beans, H. A. Fales and G. M. Ware, *Jour Amer. Chem. Soc. 41,* 487 [1919]).

Without going further into detail here, the point to be particularly emphasized is the interdependence of various lines of chemical study. Enzyme actions may be treated as a group of chemical reactions analogous to other chemical changes. The substances taking part in such actions are an integral part of chemical science. Such considerations justify the inclusion of a treatise on Enzyme Actions in a series of Monographs of Chemistry. Although the subject matter cannot be presented in as well-rounded and final(?) a form as many might desire, for others, this very incompleteness together with the fact that the topic is a living one, subject to development, growth, and change, will add interest to its future study.

The author wishes to take this opportunity to thank those who have consciously or unconsciously aided him in his studies as well as in the preparation of this monograph. He particularly desires to express his gratitude to his friends and associates, Miss Grace McGuire and Miss Helen Miller Noyes, and to his colleague Dr. I. Greenwald, all of the Harriman Research Laboratory; also to Professor Jacques Loeb and Dr. John H. Northrop of the Rockefeller Institute for Medical Research; Professors H. T. Beans and Ralph H. McKee of Columbia University; and especially to Professor John M. Nelson of Columbia University.

The author also wishes to express his appreciation to the Editor of the Series of Monographs and his associates on the Editorial Board for the uniform courtesy and kindness which they have shown, and for the advice and suggestions offered by them in connection with this monograph.

November 1, 1920.

THE CHEMISTRY OF ENZYME ACTIONS

I.—Introduction

Enzymes may be defined as catalysts produced by living matter. The study of the chemical nature of substances which occur in animal and vegetable matter and the changes these substances undergo during life processes, brought about the view that agents are present in living organisms which are capable of accelerating certain definite chemical changes in the material which is present. Without entering into the historical development of the discovery of various enzyme actions, or the relation between enzymes and ferments, it may be stated that the view has become accepted that enzymes are catalysts; that they are produced by living organisms; that they are not themselves living in that they do not possess the powers of growth and of reproduction.

Enzyme actions may be studied from various points of view. In the first place, attention may be fixed upon a certain chemical reaction and a search made for animal and vegetable materials from different sources such as tissue extracts, vegetable extracts, etc., which accelerate the velocity of the chemical change. Secondly, a definite preparation may be tested with a variety of chemical reactions in order to determine the different enzyme actions it possesses. These methods of investigation have been carried on very extensively and have yielded a rich harvest. Innumerable reactions may be listed whose velocities are modified by the addition of one or another of the enzyme preparations. This purely descriptive method of study must necessarily be the first step in the development of a science of enzyme reactions.

Following this stage of the study, two methods of attacking the problem present themselves. Enzymes, as catalysts, modify reaction velocities. A systematic study of reaction velocities, their significance, and the factors upon which they depend would be in order. Then the general problem of catalysts would be considered, and finally, enzymes as a special group of catalytic substances. The second method of attack would include the study of the chemical nature of enzymes, their compositions, structural formulas, and reactions. That is to say, enzymes would be studied as though they possessed definite chemical structures.

An attempt will be made here to indicate the progress which has been made in recent years by these two methods of attack. The descriptive methods of enzyme study have shown so many reactions which are catalyzed by enzymes, and also such a great variety of animal and vegetable products which are capable of accelerating chemical changes, that reference to the larger textbooks of physiological chemistry and to compilations of enzyme reactions may suffice for the details of such studies.

Although a great many reactions have been found to belong to the group of enzyme reactions, it is possible to classify them in a comparatively simple manner with the aid of some recent conceptions of theoretical chemistry. These theoretical views, involving the structures of chemical compounds, will be presented here as a necessary foundation for the consideration of enzyme reactions and the factors influencing their velocities.

Practically all enzyme actions deal with reactions of organic compounds. For a number of years, perhaps from 1870 or thereabouts until recently, in the teaching and treatment of chemical substances, it has been customary to consider chemistry as divided into two branches, inorganic chemistry and organic chemistry. Different methods were used in experimental work in handling and working with the substances grouped in this way, and as a result, theories were developed for one or the other of these divisions. These theories were apparently limited to the one group. For example, the theory of electrolytic dissociation in solution was not considered by some of its most active advocates to be applicable to organic compounds, while at the same time a number of organic chemists ignored this theory not only in their own experimental work, but also in their teaching and writing, even when dealing with inorganic substances.

Fortunately, in recent years, the development of chemical theory has taken a more rational trend. Theories are considered to apply to all chemical compounds. Division of substances into organic and inorganic compounds is for convenience only. This matter of convenience may refer to such practical questions as teaching, occurrence in nature, like properties, etc. A certain theory may be developed with one group of compounds. This theory apparently does not apply in the same way to a different class of compounds, but a more careful study will always show that conditions of testing may be different; that a common truth applicable in some way to both classes is present; and therefore that the theory as developed was incomplete. Thus, the theory of electrolytic dissociation was developed in connection with certain properties of inorganic substances in aqueous solution. At first, the theory was not considered to apply to organic substances either as solutes or solvents. Then further experimentation showed that organic substances, to varying extents, showed the phenomenon of electrolytic dissociation in solutions. The most recent development brings out the view that all chemical combinations between atoms are electrical in character, and that for a substance to show electrolytic dissociation in solution, the participation of a solvent with certain definite properties is essential.

With this viewpoint of chemical theory in mind, it will be possible to consider the basic theories of chemical reactions somewhat further. A general knowledge of structural organic chemistry is presupposed. Enzyme reactions, or the reactions accelerated by enzymes, may be divided into two general classes. In one class, the elements of water or of some similar compound may be added to or eliminated from the substance being changed; in the other class, oxidation or reduction occurs. The first class includes such reactions as the hydrolysis of esters, of complex sugars including di-, tri-, and polysaccharides, of urea, hydrolysis of amides, etc.

The second class of reactions mentioned, that including oxidation and reduction, may be considered somewhat more in detail. Oxidation or reduction refers in any given case to one or more definite atoms of a molecule and not to the molecule as a whole. With inorganic compounds, the view has been generally accepted that oxidation consists in an increase in the positive charge of the atom being oxidized or a decrease in the negative charge, reduction in a decrease in the positive charge or an increase in the negative charge. Using the electron conception of valence, these views become simplified and applicable to all classes of compounds. The electron conception of valence states that in the formation of a union or linking between two atoms, one of these atoms loses a negative electron and the other gains this electron. As a result of this linking, the first of these atoms acquires a unit positive charge, the second a unit negative charge. This means simply that valence instead of being denoted by a number is stated as a positive or negative number. In oxidation, the valence of the atom being oxidized is increased, in reduction, decreased. Thus, the change of ferrous chloride into ferric chloride is represented as follows:

$$\overset{++}{\mathrm{Fe}}\overset{-}{\mathrm{Cl}}_2 \rightarrow \overset{+++}{\mathrm{Fe}}\overset{-}{\mathrm{Cl}}_3$$

The valence of the iron is increased one unit, the valence of the chlorine remains unchanged. The change from hydrogen sulfide to sulfur dioxide is represented as follows:

$$\overset{+}{\mathrm{H}}_2\overset{--}{\mathrm{S}} \rightarrow \overset{+4}{\mathrm{S}}\ \overset{--}{\mathrm{O}}_2$$

Many other examples may be quoted but the significant feature is that one definite atom is oxidized or reduced, loses or gains negative charges. If the complete equation of a chemical reaction is written, then if one atom is reduced, some other atom must be oxidized or vice versa. It is necessary to have an equivalence of positive and negative charges or valences just as it is necessary to have an equivalence of atoms. The charges indicated as the valences of the atoms are only in special cases susceptible to direct experimental measurement. They are assumed to be present as a consequence of the development of valence theories and account satisfactorily for a number of facts not readily explicable without them. The same views and structures apply to organic compounds. The change of methane to carbon dioxide may be represented as follows:

$$\overset{-4}{\mathrm{C}}\overset{+}{\mathrm{H}}_4 + 2\overset{-2}{\mathrm{O}}_2 = \overset{+4}{\mathrm{C}}\overset{=}{\mathrm{O}}_2 + 2\overset{+}{\mathrm{H}}_2\overset{=}{\mathrm{O}}$$

(the oxygen molecule may be assumed to be either $\overset{++}{\mathrm{O}} = \overset{=}{\mathrm{O}}$ or $\overset{\pm}{\mathrm{O}} = \overset{\mp}{\mathrm{O}}$).

The carbon atom is oxidized eight units of valence, the hydrogen is neither oxidized nor reduced, while the oxygen of the oxygen molecule must be reduced eight units of valence. All organic oxidations and reductions may be considered similarly and bring such reactions into line with inorganic oxidations and reductions. This substitutes one explanation of oxidation-reduction phenomena for the two or more which were necessary when a distinction was made between the theories of structures of organic and inorganic compounds such as is designated at times by non-polar and polar compounds. With more or less complex organic compounds, a difficulty is met with at times in determining the atom or atoms which are oxidized or reduced, but even here, the general principles may be applied.

For the reactions of the first class involving in the main hydrolyses, the use of the electron conception of valence does not, at present, add anything essential to their consideration. There is no change in the state of oxidation of any of the atoms of the molecules taking part in the reactions.

These views of chemical structure are not apparently necessary in a treatment of enzyme reactions. While it is true that enzyme reactions might be treated by themselves without considering their relation to or bearing on other chemical phenomena, it would appear that the time has come to include enzyme reactions in the group of general chemical reactions, amenable to the same conditions and explainable by the same theories. Any general theory found useful in the consideration of other chemical reactions should therefore be considered or at least mentioned in connection with enzyme reactions. The further development of enzyme actions and reactions will unquestionably follow chemical lines. The science of chemical reactions is at present based upon certain theories, and the study of such reactions involves the application of these theories and at the same time results in the further development of these theories. There is no reason to place enzymes and their actions in a separate category. As chemical substances and chemical actions they must form a part of the chemical whole. For various reasons, the application of comparatively simple chemical relations to these actions has not been as successful heretofore as with some of the other groups of chemical substances and reactions. However, this does not make further attempts in the same direction unnecessary. It is therefore advisable to present some of the more recent viewpoints of chemical theory bearing upon these and related problems, with the hope and expectation that they will be found applicable and useful in some form. Especially in the oxidation reactions of enzymes, as will be brought out again in a later chapter, are the results at present available unco-ordinated and unsystematized. This is the apology, if such be needed, for the inclusion here of theories and hypotheses apparently not directly connected with enzyme actions, and heretofore often ignored in their treatment.

Several other theories must also be discussed briefly since the consideration of chemical actions is based to a certain extent upon them and their modifications.

The ionic theory or the electrolytic dissociation theory of Arrhenius has been extremely successful in accounting for a number of facts observed in solutions and in correlating apparently diverse phenomena. The reactions of salts in aqueous solutions were ascribed to the presence of ions whose existence has been demonstrated repeatedly. Some zealous supporters of the ionic theory even went so far as to state that only ions take part in chemical reactions. This point of view was never considered seriously. The fact that unionized molecules as well as ions may take part in chemical reactions and that frequently both may be assumed to do so was shown later by a number of workers. This question will be taken up in greater detail in Chapters III and IV.

An attempt was made a few years ago to develop these problems farther.[1] A somewhat more detailed account was published recently.[2] In brief, the view was advanced that the readiness or speed with which reactions occurred was a phenomenon not dependent upon the existence of ions. The occurrence, existence, and stability of ions in the same way had nothing to do directly with the occurrence of chemical reactions. There is, however, an indirect connection. The physical property shown by the ability to conduct the electric current in solution and the chemical property shown by the ability and readiness to undergo change in composition alone or in conjunction with other substances, are both assumed to be due to the same underlying cause. This cause, while producing both effects, need not produce both quantitively at the same rate. That is to say, under certain conditions, the physical property would be much the more marked and amenable to experiment; under other conditions, the chemical. Elsewhere the general theory was suggested and developed that chemical reactions between two or more substances depend upon the primary formation of addition compounds. This view will be spoken of more in detail in Chapter III. In the present connection it may be pointed out that it is probable that in solution, the property of the solvent of forming addition compounds with the dissolved substances is the common cause of the two sets of phenomena, physical and chemical. In aqueous solutions, compounds of the nature of hydrates, which have been shown to exist in a number of cases, may well be the cause. In some cases, such as with univalent salts in aqueous solutions, very close parallelism exists between the physical and chemical properties, with unidivalent and more complex salts, the parallelism is not obvious or does not exist at all. Quantitative proof of this theory is not at hand, but it has been found useful in the consideration of reactions and will be used here. To sum up these relations: The changes occurring in chemical reactions do not depend upon the electrolytic dissociation of the reacting substances. The chemical changes are accompanied very often by electrolytic dissociation phenomena, but the latter parallel the former (or vice versa) and do not necessarily precede or cause them. The electron conception of valence assumes the presence of electric charges on all atoms existing in states of combination. The experimental facts of electrolytic dissociation offer a method for making some of these electric charges susceptible to measurement, but electrolytic dissociation does not produce these charges on the ions due to valence combinations. This point must be clearly understood, otherwise confusion will result.

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[1] K. G. Falk and J. M. Nelson, *Jour. Amer. Chem. Soc.* 37, 1732 (1915).

[2] "Chemical Reactions; Their Theory and Mechanism," published by the D. Van Nostrand Company, New York, 1920.

The hydrogen ion concentration has been much emphasized in connection with enzyme actions. At the same time, its importance in other chemical actions may have been overlooked. In aqueous solutions, it is unquestionably one of the most important standards by which the properties of the solvent and dissolved substances may be controlled, and it is possible that it is from this point of view that this factor should be considered.

Another set of phenomena which has given rise to much discussion especially in recent years is included under the general topic of "colloid chemistry." Colloids are obviously of importance in connection with substances obtained from living matter. The point of view which will be adopted here is that colloids as chemical substances will be considered in the same way as other substances and that colloidal solutions will be assumed to possess chemical properties similar to other solutions. If apparent exceptions occur, or if phenomena predominate which are more in the background with the non-colloidal substances and solutions whose relations have been studied over a longer period of time, these exceptions and phenomena will not be designated by new names or branches of science, but will be retained under the group of relations which have not as yet been developed satisfactorily and which require further quantitative study.

Similarly, adsorption compounds will be considered to be fundamentally chemical in character. Because of such facts as amount of surface involved and physical state of subdivision of the reacting components, the laws of definite and multiple proportions cannot in most cases be proven to apply to the masses in question, and equilibrium between the various components may not be attained in many cases. The evidence which is rapidly accumulating, however, indicates that chemical compounds are formed with the limitations as to their extent just referred to, and that the same general laws of combination exist here as with other chemical compounds.

Some recent work by Langmuir and by Harkins on the orientation of molecules in surface films is of interest in this connection. The point of view may be shown best by a quotation from a paper by Harkins:[3] "In this paper data will be presented for the work done when the surfaces of two liquids come together to form an interface. The numerical value of this work in ergs per square centimeter is characteristic of each class of compounds, and the data show in a very striking manner that the film of any liquid in contact with water is composed of molecules oriented so that the active (or polar) group at the end of any hydrocarbon chain is in contact with the water. . . . It will be shown that the attraction between water and another liquid is one of the important factors in the determination of the solubility of the other liquid in water." The groups most active toward the water surface, or, as Harkins calls them, the very polar groups, were shown to be CO_2H, CO, CN, OH, and $CONH_2$.

The conclusions of Langmuir [4] are essentially the same except that he speaks of chemical combination between certain of the groupings or atoms of the substances composing the surfaces. Although he considers primary and secondary valences, in a sense following Werner, and is not entirely clear as to these, the crux of his theory is perfectly definite and may be given best in his own words: [5] "From the viewpoint adopted in the present paper the forces involved in adsorption, surface tension, etc., are strictly chemical in nature, that is, they do not differ in any essential respect from the forces causing the formation of typical chemical compounds."

These views are presented as they show the most recent trend which the studies on adsorption and surface actions are taking. The general conclusion appears to be that the forces acting are chemical in character and must be treated in the same way as other chemical actions. To the organic chemist especially, the results of Langmuir and of Harkins are of interest from another point of view. Those groups which are found to be most active in adsorption and surface phenomena, and combine chemically in such surfaces, are the same groups which the organic chemist has found to act most readily with chemical reagents. The organic chemist is able to bring about chemical reactions with specific atoms or groups of atoms in a more or less complex organic molecule by a suitable choice of reagents and conditions. The physical chemist has now shown how to measure by purely physical means the action of an atom or group of atoms in a complex organic molecule. The orientation by means of which it has been made possible to make these measurements (of surface tension) is based upon a chemical reaction of the atom or group of atoms in question with the second surface and is based therefore upon the same underlying properties which the organic chemist studies when he determines the "reactivity" of a grouping. The active groups of the physical chemist and the organic chemist are the same, and this method of determining such groups promises to be of value in the future study of organic compounds, whether these be simple in composition, or of complex nature such as are present in biological material.

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[3] Cf. W. D. Harkins and co-workers, *Jour. Amer. Chem. Soc.* *39*, 354, 541 (1917); *41*, 970 (1919); *42*, 700 (1920).

[4] I. Langmuir, *Met. Chem. Eng.* *15*, 468 (1916); *Jour. Amer. Chem. Soc.* *38*, 2221 (1916); *39*, 1848 (1917); *40*, 1361 (1918).

[5] I. Langmuir, *Jour. Amer. Chem. Soc.* *39*, 1848 (1917).

II.—Velocities of Chemical Reactions

The actions of enzymes manifest themselves by the changes in velocities of chemical reactions. Before entering into the specific enzyme actions, it would be well to review what is meant by velocity of a chemical reaction, the formulations and equations which are used, the significance of the various terms of such equations, the factors which may limit such formulations, and the chemical conclusions which may be derived from kinetic considerations.

The kinetic developments have been based in the main upon the number of molecules which react or are changed in the reaction under consideration. Thus the mathematical expressions which are derived depend upon whether one molecule is undergoing change as in a "monomolecular" reaction, whether two as in a "bimolecular" reaction, three as in a "termolecular" reaction, etc. Such expressions evidently depend upon a knowledge of the chemical equation of the given reaction. While this is true to a certain extent, the mathematical expressions may show that the chemical equation does not represent the change as it occurs; that is to say, either the mathematical equation or the chemical equation or both are incomplete. The mechanisms of a number of reactions have been cleared up in this manner.

It is obviously impracticable to give here a complete review of the subject of chemical kinetics. Only some of the salient features and those which may be of direct interest in enzyme actions will be considered.

The law of mass action forms the basis of the exact study of chemical kinetics. This law states that the amount of substance undergoing change in a unit of time is proportional to the active mass present during that time. This law is of general applicability. In applying it in chemical reactions, it is obviously necessary to use certain units of mass or quantity in order to determine the active mass of substance present at any given time. The simplest view to take is that the active mass of a substance is given by its molecular concentration. For practical purposes, therefore, the number of gram molecules, or mols per liter of volume will be used as the active mass.

Before going further, however, it is necessary to emphasize the simplification which has been introduced. The active masses have been replaced by molecular concentrations, and therefore the law of mass action has been changed to the law of concentration action. If, now, deductions from the law of concentrations are found not to be valid, this does not mean that the law of mass action does not hold, but that an incorrect hypothesis may have been introduced in the substitution of concentrations for active masses.

In the simplest case in which one substance is undergoing change, if the initial concentration of this substance is denoted by a, and at the end of time t, x gram molecules of a have been transformed, $a - x$ remaining unchanged, then the law of concentration action requires that, the temperature remaining constant,

$$\frac{dx}{dt} = k\ (a - x) \qquad (1)$$

By integration, and evaluating the integration constant by putting $t = 0$,

$$k = \frac{1}{t} \log_e \frac{a}{a - x}, \qquad (2)$$

or when x_1 and x_2 denote the amounts of substances transformed after the time intervals t_1 and t_2,

$$k = \frac{1}{t_2 - t_1} \log \frac{a - x_1}{a - x_2} \qquad (3)$$

These equations for a monomolecular reaction have been applied to a number of reactions and a constancy of k demonstrated. For example, in the transformation of dianthracene ($C_{28}H_{20}$) into anthracene ($C_{14}H_{10}$) according to the chemical equation

$$C_{28}H_{20} = 2C_{14}H_{10}$$

the following results were obtained at 152° in phenetol as solvent.[1]

t	0	865	1225	2345	3845	5310
D	16.38	9.35	7.03	3.26	1.18	0.55
$k \times 10^4$		1.2	1.3	1.3	1.3	1.2

In these results, t represents the time in minutes from the beginning of the reaction, D the milligrams per liter of dianthracene present at time t, and $k \times 10^4$ the monomolecular reaction velocity constant as calculated by means of equation (3) from each pair of determinations.

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[1] R. Luther and F. Weigert, Sitz. ber. Kgl. pr. Akad. Wiss. Berlin *1904*, 828.

It is evident that the value of the constant shows that the reaction follows the indicated law.

Equation (2) may also be written in the form

$$\frac{a - x}{a} = e^{-kt} \qquad (4)$$

or the substance is transformed at a rate which varies in an exponential manner with the time.

Now k in these equations represents a constant characteristic for the reaction being measured under the given conditions. Its physical significance is the rate of transformation of unit concentration of the substance (from equation (1)). Its numerical value is independent of the original concentration (a) of the substance undergoing change.

Several further derivations from equation (2) have been made and are in use especially with radioactive changes. The term $1/k$ may be called the period of average existence of the substance undergoing change and denotes the time which would be required for the substance to be transformed completely if the same amount of substance being transformed at the instant in question continued to be transformed.

The period of half change, or time required for half of the substance to be transformed, is found to be

$$t = 0.6932 \; \frac{1}{k}$$

This period of time will be the same independent of the amount of substance with which the calculation is made. It is therefore a useful constant for reactions obeying the monomolecular law.

The chemical changes involved in a monomolecular reaction lead to some interesting conclusions with regard to the reacting substances. In these reactions only one substance is undergoing change. It follows, since the whole amount of substance is not transformed instantaneously, that the substance must be present in different states or conditions. Various attempts have been made to account for these differences. Arrhenius denoted the difference by speaking of active and passive molecules and considering an equilibrium to exist between the two types. The kinetic theory, involving different velocities of translation, has been invoked, as well as vibratory or cyclic motions of the atoms within the molecule. Recently, W. C. McC. Lewis has developed an explanation involving infra-red radiations and the quantum theory of energy, which promises to throw considerable light upon this problem as well as upon chemical reactions in general.[2]

Undoubtedly a satisfactory theory will be developed in time, to account for these relations of monomolecular reactions. For the present, none of the suggested explanations will be given, but only the fact will be used, that in a monomolecular reaction, the velocity of transformation is proportional to the concentration.

In a bimolecular reaction two substances are undergoing change simultaneously. Since it is probable that the amount of reaction is proportional to the number of times the particles of the two substances meet, the total change at any instant would be proportional to the product of the concentrations (or active masses) of the substances undergoing change. Similar methods of formulation as for monomolecular reactions are in use. Starting with *a* gram molecules of each of the reacting substances, if after time *t*, *x* gram molecules of each have reacted, then the velocity of the reaction will be represented by the equation

$$\frac{dx}{dt} = k\ (a - x)^2, \tag{4}$$

which on integration becomes

$$ak = \frac{1}{t} \cdot \frac{x}{a - x} \tag{5}$$

in which *ak* is constant.

Starting with different amounts, *a* and *b* of the two products, the equations assume the following forms:

$$\frac{dx}{dt} = k\ (a-x)\ (b-x) \tag{6}$$

$$(a-b)\ k = \frac{1}{t} \log_e \frac{a(b-x)}{b(a-x)} \tag{7}$$

The usual example of a bimolecular reaction is the hydrolysis of ethyl acetate by sodium hydroxide

$$CH_3CO_2C_2H_5 + NaOH = CH_3CO_2Na + C_2H_5OH.$$

With equivalent amounts of reacting substances, the following results have been obtained (equation 5) and are generally quoted:—[3]

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[2] **W. C. McC. Lewis, "A System of Physical Chemistry," Vol. 3, pp. 141-3 (1919);** *J. Chem. Soc.* ***105***, 2330 (1914); ***107***, 233 (1915); ***109***, 55, 67, 796 (1916); ***111***, 389, 457, 1086 (1917); ***113***, 471 (1918); ***115***, 182, 710, 1360 (1919); ***117***, 623 (1920); ***Phil. Mag. 39***, **26 (1920);** ***Scientia, 25***, No. LXXXVI—6, June, 1919.

[3] **R. B. Warder, Ber.** ***14***, **1361 (1881);** ***Amer. Chem. Jour. 3***, **340 (1882).**

t	x	ak
5	5.76	0.113
15	9.87	0.107
25	11.68	0.108
35	12.59	0.106
55	13.69	0.108
120	14.90	0.113

With amounts of reacting substances not equivalent, the following results may be quoted[4] (equation 7):

	Alkali in Excess				*Ester in Excess*		
t	(a—x)	(b—x)	(a—b)k	t	(a—x)	(b—x)	(a—b)k
0	0.5638	0.3114	——	0	0.3910	0.6593	——
393	0.4866	0.2342	0.0335	342	0.2885	0.5568	0.0346
669	0.4467	0.1943	0.0342	670	0.2239	(0.4222)	0.0347
1010	0.4113	0.1589	0.0339	888	0.1925	0.4605	0.0345
1265	0.3879	0.1354	0.0346	1103	0.1677	0.4350	0.0344

These examples will suffice to show the use of the equations, although many other reactions have been found to follow the bimolecular law.

The experimental results which have been given to illustrate mono- and bimolecular reactions show that the kinetic laws hold for these cases. A number of further reactions which might be quoted show in the same way the apparent validity of the deductions. However, to assume that the kinetic laws hold for every reaction under varying conditions is not permissible. In fact, it is not even permissible to extend the relations for any one reaction beyond the limits where the law has been found experimentally to hold. Increasing the accuracy of the experimental work and also modifying the conditions, for example by the addition of a neutral salt, has resulted in several cases in changing the value of the constant, which had under different conditions shown constancy, so that the kinetic equations could no longer be used satisfactorily. This question will be taken up again in Chapter IV. For the present, it may be stated that when satisfactory values for the velocity constants have been obtained, these values should not without further evidence be applied to the reaction under modified conditions, unless the changes in the conditions and the influences these conditions exert upon the substances and their concentrations, whether these be calculated as molecules or ions, are carefully scrutinized. It is possible that at times assumptions have been introduced into the calculations in this way, and that apparent lack of agreement of the kinetic with the molecular equations is due to incorrect designation of the molecular species or their concentrations which are involved in the reaction.

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[4] L. T. Reicher, Lieb. Ann. *228*, 257 (1885); *232*, 103 (1886).

With three substances taking part in the reaction, starting with the same initial concentrations, the equations assume the following forms:

$$\frac{dt}{dx} = k\ (a-x)^3 \tag{8}$$

$$k = \frac{1}{t} \cdot \frac{1}{2}\left(\frac{1}{(a-x)^2} - \frac{1}{a^2}\right) \tag{9}$$

Here *k* is inversely proportional to the square of the initial concentration of the reacting substances.

In general, the equations for an *n*-molecular reaction are as follows:

$$\frac{dx}{dt} = k\ (a-x)^n \tag{10}$$

$$k = \frac{1}{t} \cdot \frac{1}{n-1}\left(\frac{1}{(a-x)^{n-1}} - \frac{1}{a^{n-1}}\right) \tag{11}$$

The number of reactions which obey the equations of higher orders decreases with the number of substances taking part in the reactions. From kinetic considerations such a result would follow from the probability of the smaller number of meetings of a greater number of molecules with resulting reaction.

The equations which have been given form the basis for chemical kinetics. Since in this book, it is intended only to outline such relations, the reader is referred to other works where these velocity equations are treated more in detail and more examples quoted. The methods which have been suggested for determining the order of a reaction will not be gone into here.

The further considerations will take up questions which have been found to be of interest in connection with enzyme and similar reactions.

The velocity equations refer in any given case to a definite constant temperature. Increase in temperature will increase the velocity of reaction or the value of *k*. It has been found experimentally that an increase of 10° in temperature will increase the value of *k* or the velocity of a chemical reaction two to three times at temperatures in the neighborhood of those used ordinarily. Empirical equations have been proposed for this large increase in velocity with rise in temperature, but up to the present there appears to be no satisfactory explanation for the phenomenon.

The value of *k* is characteristic for a given reaction at a definite temperature. Any change in the reaction mechanism or the conditions under which the reaction is taking place would show itself in a changing value of *k*. This fact will be taken up again.

If one of the reacting substances is present in large excess so that in the chemical reaction taking place its concentration remains practically unchanged, then this concentration may be considered to be constant in the velocity equation and the order of the reaction found will be smaller than that actually occurring. Thus, in the hydrolysis of sucrose according to the equation

$$C_{12}H_{22}O_{11} + H_2O = C_6H_{12}O_6 + C_6H_{12}O_6$$

the velocity of the reaction should be of the second order. In dilute solution, the concentration of the water remains practically unchanged so that the velocity of the reaction may be found to be proportional to the concentration of sucrose. This reaction is therefore often considered to be a monomolecular reaction.

The chemical reactions have been considered so far as proceeding in one direction only. As a matter of fact few reactions in homogeneous media do this. The products formed tend to recombine to form the original substances with a velocity of their own. Similar velocity equations can therefore be set up to represent these reverse reactions. After a certain time these reactions in opposite directions will proceed at such a rate that the composition of the mixture will remain unchanged; that is, equilibrium will have been attained. This equilibrium will be kinetic, not static, reaction still continues, but in opposite directions so that the chemical composition of the mixture is constant. The kinetic equations to represent such an equilibrium will evidently consist of the difference in the velocities of the two reactions.

In equation (10), denoting the reverse reactions as follows:

$$\frac{dx}{dt} = k_1(a_1-x)^{n_1} \text{ and } \frac{dx}{dt} = k_2(a_2-x)^{n_2}, \qquad (12)$$

then for equilibrium to exist,

$$k_1(a_1-x)^{n_1} = k_2(a_2-x)^{n_2}, \text{ or}$$

$$\frac{k_1}{k_2} = \frac{(a_2-x)^{n_2}}{(a_1-x)^{n_1}} = K \qquad (13)$$

in which K represents the equilibrium constant of the reaction at a definite temperature. Both k_1 and k_2 vary with the temperature and not as a rule at the same rate; therefore K will also vary with the temperature but need not increase as the velocity constants do with rise in temperature. This method of developing the significance of the equilibrium constant must retain in that term the limitations which are inherent in the velocity constants. The factor of the law of concentrations enters here. Another method of developing the equilibrium constant is from the thermodynamic side without using the concepts of reaction velocities. This method, however, has similar limitations in that the simple laws of dilute solutions (or of gases) are assumed to hold for each constituent.

Equation (13) brings out another fact. If a condition of the reaction in one direction is changed so as to increase its velocity, but at the same time does not change the value of the equilibrium constant, then the velocity of the reaction in the opposite direction must be increased correspondingly. This is of interest in connection with catalysts as will be brought out in a later chapter.

Evidence is accumulating steadily to the effect that the chemical equations as written ordinarily do not represent the actual course of reactions. In some apparently complex reactions, further study has revealed the fact that two or more successive simple reactions go to make up the more complex change. If, in studying the kinetics of such a change, one of these simple reactions proceeds at a much slower rate than the others, this will be the reaction whose velocity is measured in place of the complete complex change. If the velocities of the separate consecutive reactions are more nearly equal, then the mathematical treatment is more complicated. For example, in the simplest case of two consecutive monomolecular reactions, A → B → C, starting with a mols of A, after the time t, x mols of A, y mols of B, and z mols of C are present. Then

$$x + y + z = a \tag{14}$$

Also, if k_1 is the velocity constant of the reaction A → B, and k_2 of the reaction B → C, then

$$-\frac{dx}{dt} = k_1x, \tag{15}$$

$$\frac{dz}{dt} = k_2y, \tag{16}$$

and
$$\frac{dy}{dt} = -\frac{dx}{dt} - \frac{dz}{dt} = k_1x - k_2y. \tag{17}$$

On integration
$$a-z = a\left(\frac{k_2}{k_1-k_2}\,e^{-k_1t} + \frac{k_1}{k_2-k_1}\,e^{-k_2t}\right). \tag{18}$$

In order to use these equations certain simplifications are necessary. This has been done for a number of cases. Reference will be made to these consecutive reactions again.

The question of reactions in heterogeneous systems is of importance. As a comparatively simple case, the solution of a solid in a liquid may be considered. In reality this reaction or process consists of two consecutive reactions: (1) a reaction between solid and solvent, and (2) the diffusion away of the products of the reaction. In general terms, if the concentration of the (saturated) solution in immediate contact with the solid is denoted by a, the concentration of the rest of the solution at any time t by x, and s the area of the surface, then the rate of solution is given by the equation
$$\frac{dx}{dt} = ks\,(a-x), \tag{19}$$

and on integration
$$ks = \frac{1}{t}\log_e\frac{a}{a-x}. \tag{20}$$

The surface s is taken to be constant. This equation has been found to hold for the solution of benzoic acid in water, etc.[5] With the first action extremely rapid, the velocity of the reaction is given by the rate of diffusion of the saturated solution into the rest of the liquid. W. Nernst[6] extended this conception to include all reactions occurring in heterogeneous systems. He considered that the equilibrium at the surface of two phases is set up very rapidly, practically instantaneously, in comparison with the velocity of diffusion. This view has been applied to a number of different reactions. Since the equation representing the velocity of diffusion is similar in form to the equation of a monomolecular reaction, whenever the latter appears to hold for a reaction taking place in a heterogeneous system, it is probable that the reaction velocity measured is that of a rate of diffusion. This applies to reactions occurring on surfaces, and in general to catalytic reactions taking place in more than one phase.

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[5] A. A. Noyes and W. R. Whitney, *Z. physik. Chem.* **23**, 689 (1897).

[6] *Z. physik. Chem.* **47**, 52 (1904).

This simple view was extended by Fink,[7] who showed that in the chanism of heterogeneous reactions, the reaction velocity is limited the rate of diffusion of the reacting substances to the surface where reaction is taking place through an adsorbed film of variable thick ss of the substances taking part. I. Langmuir[8] as a result of the dy of reactions of gases at very low pressures on metals developed s theory further and applied it to reactions in heterogeneous sys ns in general. It will not be possible to enter into the details of ese interesting relations at present. They are mentioned here since zyme reactions frequently take place in heterogeneous systems and is possible that some form of the theories developed will be found plicable to the kinetics of the actions.

As stated this relation is important for enzyme actions since zymes occur as colloids. Care must therefore be exercised from this int of view in determining the velocities of enzyme reactions. Just w far colloids may be considered to be in homogeneous phase in ueous solution is an open question at present. Every case must be nsidered on its own merits, but the possibility of the velocity of the action measured not being that of the supposed chemical reaction ist always be kept in mind.

Reference may be made to the velocities of reactions represented radioactive changes. These changes all follow the monomolecular action velocity law and represent a number of consecutive reac ns. The fact that a change of state occurs in most of these trans rmations raises the question whether the velocity measured is not diffusion velocity of one or more of the products from the seat of reaction, similar to other heterogeneous reactions. The action of enzyme, catalase, may be mentioned in this connection.

The kinetic equations have been applied to chemical reactions so in a more or less simple manner with apparently satisfactory re lts. However, it has been found that more careful investigations ve brought to light facts which did not agree with the simple laws kinetics to which they apparently conformed. Thus, the hydrolysis esters, as catalyzed by acids, is not entirely a simple reaction (cf. . J. Jones and A. Lapworth[9]). The hydrolysis of sucrose by acids, first reaction in which the law of mass action was used (by Wilhelmy[10] in 1851) on more careful study in recent years, still requires further elucidation.

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[7] C. G. Fink, Dissertation, Leipzig, 1907; M. Bodenstein and C. G. Fink, *Z. physik. em.* **60**, 1, 46 (1907).

[8] I. Langmuir, *Jour. Amer. Chem. Soc.* **37**, 1139 (1915).

[9] *J. Chem. Soc.* **99**, 917 (1911).

Part of the exceptions to the simple kinetic equations may be found in the use of concentrations in place of "active masses." The simple laws of chemical kinetics apply only in dilute solutions or at concentrations where the gas laws hold. Strictly speaking, the concentration term holds throughout a given reaction if the relation between concentration and active mass does not change. For pure substances, the vapor pressure gives a measure of the active mass, and if the change in vapor pressure, the active mass, and the concentration run parallel, then the kinetic deductions hold. These relations hold for the equilibrium constants of chemical reactions, whether these be derived from kinetic or thermodynamic standpoints. In both cases, limitations of concentration must be introduced and the simplest criterion is that supplied by the gas laws.

It follows, therefore, that if the kinetic equations do not conform to the chemical equations, the conditions under which the kinetic equations are applied must be carefully scrutinized. There is at present no reason to assume that the law of mass action does not hold, but the method of applying it, especially in concentrated solutions, requires further study.

At the same time, the other possibility must be kept in mind; namely, that the kinetic equation does not represent the change taking place in the chemical equation. As a rule, the chemical equation is obtained by means of the chemical analyses of the initial and final substances taking part in the reaction. If these are definite, then the simple application of the velocity equations is permissible. Even so, it is conceivable and indeed very often probable, that the reaction takes place in stages and that the velocity which is being measured is that of the slowest reaction. In fact, a general theory of chemical reactions will be presented in the next chapter which includes this view as a necessary consequence for reactions of higher orders. Even with such a limitation, the application of velocity equations will aid in throwing light on a number of reactions. If, however, a chemical reaction does not take place in a simple manner, but on the contrary results in the formation of a number of different products, then the information which may be obtained from the velocity equations will be more limited. Such reactions have been treated from the kinetic standpoint as simultaneous reactions by

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[10] L. Wilhelmy, Pogg. Ann. *81*, 413, 499 (1851).

Wegscheider[11] and others, and the criteria for their presence formulated. They form another special group of velocity equations, and while of interest from the kinetic standpoint, are not at present useful in the consideration of enzyme actions. They will not, therefore, be considered further here.

The basic principles of the kinetics of chemical reactions have been given in this chapter because the dominating feature of an enzyme action is shown as a change in the velocity of a definite chemical reaction. It will be seen in later chapters that these kinetic equations only hold in exceptional cases of enzyme actions. The question may therefore be asked, why are they given? Here again, the point of view brought out in the first chapter must be emphasized. The kinetic relationships of enzyme actions must be treated in the same way as the kinetic relationships of chemical actions in general. Based upon the same fundamental factors, the practical application of the simple equations may well be different in different chemical reactions because of the conditions peculiar to each reaction. These conditions may be such as to necessitate additional assumptions in applying the kinetic equations to the chemical equations, or such as to necessitate modifications of experimental conditions to permit the use of the kinetic equations, or still other relations. In any event, the ultimate application of chemical kinetics to enzyme reactions will be based upon and developed from the general principles of chemical kinetics outlined superficially in this chapter, even if the useful applications seem somewhat remote. This part of the problem will be developed further in Chapter VIII, and some of the applications of kinetic relationships to enzyme reactions shown.

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[11] R. Wegscheider, *Monatsh. f. Chem.* 22, 849 (1901).

III.—General Theory of Chemical Reactions; Catalysis

In the last chapter the velocity equations which are in use to represent the rates of chemical reactions were outlined, as well as some of the factors which must be taken into account in the application of these equations. In this chapter a general theory of the mechanism of chemical reactions will be outlined and the relation which catalytic reactions as a special group bear to the general theory indicated.

Before proceeding to this, however, a brief outline of some of the work and the views of others on catalysis will be given. This is essential since enzyme actions are treated as a rule as a group of catalytic actions, and a more general survey of the field of catalytic action is desirable. It will be impossible to enter into details, but sufficient references will be given to enable those interested to go further.

Berzelius[1] was the first to group certain reactions in a general class as catalytic. He considered a catalytic agent to be "a substance which merely by its presence and not through its affinity, has the power to render active affinities which are latent at ordinary temperatures." During the following sixty years, a great number of reactions were described as belonging to the group of catalytic actions, and gradually certain added empirical relations were developed as criteria for a reaction to be considered catalytic; such as, the catalytic agent remaining unchanged, a small amount of catalytic agent transforming large amounts of substances, the equilibrium of the reaction being unchanged, etc. Ostwald[2] in 1902 classified and systematized catalytic reactions. He defined catalysis as the acceleration of a reaction by a substance which does not appear in the products of the reaction. An interesting account of the development of catalytic views and the important part which Ostwald played in this connection can be found in his Nobel prize address delivered in 1909 on the subject "Ueber Katalyse."

[1] J. Berzelius, *Jahresberichte 13*, 237 (1836); *20*, 452 (1841); *Ann Chim. Phys. (3) 61*, 146 (1836); *Lehrbuch d. Chem. 3te Aufl. 6*, 19-25 (1837).

[2] W. Ostwald, "Ueber Katalyse," Leipzig, 1902; *Lehrb. d. Allgem. Chem. 2te Aufl.* (1903) p. 515.

G. Bredig[3] in 1902 published a very complete review of catalytic actions and the chemical bases underlying the reactions, taking catalysis as the acceleration (possibly negative) of a slow chemical reaction by the presence of a foreign body.

The work on catalysis and the theories developed up to this time are summarized and described and numerous references given by J. W. Mellor[4] in his "Chemical Statics and Dynamics" to which the reader may be referred.

A large amount of material has been accumulated in more recent years with regard to catalytic actions and some viewpoints of general interest developed. Some of these will be mentioned in connection with the discussion of the chemical reactions catalyzed by enzymes. However, the theoretical and experimental researches of J. Stieglitz and his co-workers must be referred to before going on to the point of view which is to be used here. Stieglitz studied the saponification of imido esters (as experimentally more favorable material than the saponification of ordinary esters) and also extended his work to compounds more or less related, such as urea ester salts, etc. He considered acceleration (possibly negative) as the only fundamental property characteristic of a catalytic action by chemical reagents,[5] further developments being based upon the fundamental laws of chemistry. The catalytic actions in the reactions studied were shown to be due to increase (or decrease for retardation) in concentration of the ions or other components which reacted. The important generalization was pointed out[6] that "a general tendency is exhibited for the positive ion of a weaker base to go over into the positive ion of a stronger base (e.g. an imido ester ion into the ammonium ion), suggesting some kind of electric potential or loss of free energy as a 'driving force'."

It will be necessary to take up somewhat more in detail now one of the factors of the kinetic equations discussed in the last chapter. The constant *k* of the reaction velocity equations is a constant characteristic for the chemical reaction in question under certain conditions. In every case *k* represents the amount of change in unit time starting with unit concentration and keeping the concentration constant throughout the unit of time. The effect of temperature was given in the last chapter. For a chemical reaction, the value of *k* is increased two or three times for an increase of 10 degrees in temperature. The rate of diffusion for a similar rise in temperature is very much smaller, so that this has been used to determine whether certain measured velocities were due to chemical reaction or to diffusion.

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[3] *Ergebnisse der Physiologie I*, 134-212 (1902).

[4] Published in 1904. Cf. especially Chapter X.

[5] ***Proc. Congr. Arts and Sciences*, St. Louis, 1904, Vol. IV, pp. 276-84.**

[6] *Am. Chem. J.* *39*, 418 (1908); *Jour. Amer. Chem. Soc.* *32*, 225 (1910).

Now, k is assumed to be constant if conditions do not change. It may be asked what is meant by conditions which do not change. In a reaction taking place in a gaseous system, it has been found that the presence of an indifferent gas, that is, one which does not take part in the reaction, does not change the value of k, the volume being kept constant. In a solution, however, matters are different. If a reaction is studied in a number of different solvents, it is found that the velocity at a definite temperature and concentration may vary widely. Perhaps the best example of this is shown by the reaction between triethylamine and ethyl iodide to form tetraethylammonium iodide, studied by N. Menschutkin.[7] One volume of the mixture of the two reacting substances was heated with 15 volumes of the solvent in a sealed tube at 100 degrees for definite lengths of time. The reaction in every case followed the equation for the bimolecular law, but the values of the velocity constant k were different with the different solvents, ranging from 0.000180 to 0.133 as shown in the following table.

The ratios given in the last two columns show the relative rates of reaction, first when compared to the slowest reaction, whose velocity is placed equal to one, and second, on the basis of the most rapid reaction which is placed equal to 100.

Velocity of Combination of Triethylamine with Ethyl Iodide in Various Solvents

HYDROCARBONS	VELOCITY CONSTANTS	RATIOS	
Hexane C_6H_{14}	0.000180	1	0.13
Heptane C_7H_{16}	0.000235	1.3	0.17
Xylene $C_6H_4(CH_3)_2$	0.00287	15.9	2.2
Benzene C_6H_6	0.00584	38.2	4.4
HALOGEN COMPOUNDS			
Propyl chloride C_3H_7Cl	0.00540	30	4.0
Phenyl chloride C_6H_5Cl	0.0231	128	17.4
Phenyl bromide C_6H_5Br	0.0270	150	20.3
Bromnaphthalene $C_{10}H_7Br$	0.1129	627	84.9

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[7] *Z. physik. Chem.* **6**, 41 (1890).

SIMPLE ETHERS			
Ethyl isoamyl ether $C_2H_5OC_5H_{11}$	0.000630	3.5	0.47
Ethyl ether $C_2H_5OC_2H_5$	0.000757	4.2	0.57
Phenetol $C_2H_5OC_6H_5$	0.0212	117.7	16.0
Anisol $CH_3OC_6H_5$	0.0403	223.9	30.3
ESTERS			
Isobutyl acetate $C_4H_9.O.CO.CH_3$	0.00577	32.1	4.3
Ethyl acetate $C_2H_5O.CO.CH_3$	0.0223	123.9	16.7
Ethyl benzoate $C_2H_5O.CO.C_6H_5$	0.0259	143.9	19.4
ALCOHOLS			
Isobutyl alcohol $C_4H_9.OH$	0.0258	143.3	19.4
Ethyl alcohol $C_2H_5.OH$	0.0366	203.3	27.5
Allyl alcohol $C_3H_5.OH$	0.0433	240.5	32.5
Methyl alcohol $CH_3.OH$	0.0516	286.6	38.0
Benzyl alcohol $C_6H_5.CH_2.OH$	0.133	742.2	100.0
KETONES			
Acetone $CH_3.CO.CH_3$	0.0608	337.7	45.7
Acetone (14.5 vol.) + water (0.5 vol.)	0.0889	493.9	66.9
Acetophenone $CH_3.CO.C_6H_5$	0.1294	718.7	97.3

The most obvious explanation of these results is that the solvent takes part in the reaction in some way. The replacement of part of a solvent by another, such as water by alcohol, changes the velocities of reactions taking place in them considerably. It would appear that it is possible to go further and to consider that the addition of any substance to a mixture or to a solution may change the velocity of a reaction. Also, in the course of a reaction, the formation of the products and the disappearance of the reacting substances may be enough to affect the value of the velocity constant to a considerable extent. The next step in these considerations would include the cases where very small amounts of added substances change the velocities of chemical reactions to a very marked degree, and this is the region in which the actions of catalysts are placed. In looking at reactions in this way, it appears as if these extreme cases, from the complete change of solvent to the addition of very small quantities of certain substances, because of the existence of all the intermediate steps in which the velocities of chemical reactions are altered, must be explainable or accounted for by the same underlying cause.

An attempt will now be made to show a probable cause for the different phenomena described in the last paragraph. One general explanation is taken to underlie the facts. A more detailed account of this explanation has been presented elsewhere.[8] Only a few of the salient points will be mentioned here.

The theory of chemical reactions which will be used is generally known as the addition theory. According to this theory, when two or more molecules react, the first step consists of the formation of an addition compound between these molecules. This addition compound then reacts further to form the products which are actually observed.

In any given case, two or more products may react to form a complex addition compound. The latter may then in turn react further to form a variety of different products. Thus, the reaction between ammonia, hydrogen chloride, and water may be formulated as follows:

$$\left[\begin{array}{c} NH_3 \\ HCl \\ H_2O \end{array}\right] \begin{array}{ll} = NH_3 + HCl + H_2O & (a) \\ = NH_4Cl + H_2O & (b) \\ = NH_4OH + HCl & (c) \\ = NH_3 + HCl.H_2O & (d) \end{array} \quad \text{I}$$

In this formulation, the complex addition compound is indicated by the components within the brackets, but the actual linkings of this compound are not indicated. Starting with products (a), the addition compound is formed which may then go back to (a) or to (b), (c), or (d). The reaction actually observed will depend upon the equilibrium constants of the four separate reactions if equilibrium is attained, or the concentrations of the various components and the relative reaction velocities if equilibrium is not reached. The reaction between ammonia, hydrogen chloride, and platinic chloride might be formulated similarly:

$$\left[\begin{array}{c} 2NH_3 \\ 2HCl \\ PtCl_4 \end{array}\right] \begin{array}{ll} = 2NH_3 + 2HCl + PtCl_4 & (a) \\ = 2NH_4Cl + PtCl_4 & (b) \\ = (NH_3)_2PtCl_4 + 2HCl & (c) \\ = H_2PtCl_6 + 2NH_3 & (d) \end{array} \quad \text{II}$$

In this reaction, the possible action of water is omitted. As written, platinic chloride takes the place of the water in I. The reaction between an alcohol and an acid in the presence of a catalyst such as hydrogen chloride would be formulated as follows:

$$\left[\begin{array}{c} C_2H_5OH \\ CH_3CO_2H \\ HCl \end{array}\right] \begin{array}{ll} = C_2H_5OH + CH_3CO_2H + HCl & (a) \\ = CH_3CO_2C_2H_5 + H_2O + HCl & (b) \\ = C_2H_5Cl + CH_3CO_2H + H_2O & (c) \end{array} \quad \text{III}$$

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[8] **"Chemical Reactions; Their Theory and Mechanism."**

A number of other equilibria are possible in this case, but those given illustrate the method. In every case, the products actually observed depend upon the principles outlined. In reaction III, products (c) may be obtained if a tertiary alcohol is used.

The theory of chemical reactions outlined evidently introduces complications into the treatment from the standpoint of reaction velocity. It is possible that the lack of success which has attended so many of the attempts to apply the kinetic equations to chemical reactions is due to this cause. The decomposition of the addition compound in the various ways in any one case is characteristic for that compound and for the given conditions. The amounts of products obtained from this addition compound in any one equilibrium equation will naturally depend upon the concentrations of the substances which may be formed and the velocity of the reverse reaction. If the velocity of the decomposition of the addition compound alone is measured, then the reaction will obey the monomolecular law; if the reaction is made up of two of the equilibria given, and if one of the reactions takes place much more rapidly than the other (the one reaction forming the addition compound, the other decomposing it), then the velocity observed will be that of the slower reaction. For reactions with inorganic compounds it is probable, in general, that with a given set of conditions and starting with certain substances, the addition compound is formed and then reacts further mainly according to one definite course. With organic compounds, on the other hand, there are, as a rule, a greater number of possibilities of reactions, and consequently a greater number of equilibria, and, unless one reaction predominates, a more complex formulation of the kinetics of the changes.

A certain awkwardness is apparent in the descriptions of the mechanism of the reactions involving a complex intermediate compound. In the formulations of the different possibilities of a reaction, at times equilibria are spoken of without the intention of conveying the meaning that the various substances taking part are present at definite equilibrium concentrations. The term equilibrium is used in these cases in place of the more usual "chemical equation" to emphasize the significance of reversibility and mass action effect.

It was suggested that a catalytic reaction be defined as a reaction in which the chemical composition of one of the initial and final products is the same. This product is known as the catalyst. In reaction I, equilibria (a) and (b), water would be the catalyst in the formation of ammonium chloride from ammonia and hydrogen chloride. A catalyst is generally assumed to increase the velocity of the reaction. However, in a number of cases, retardations have been observed when small amounts of certain substances were added, and the phenomena termed negative catalysis.[9] Also a small quantity of the catalytic substance is supposed to be able to accelerate the changes in large amounts of the other reacting substances. Both these relations are included in the view of catalysis given here. If a reaction between two substances to form two other substances takes place at a definite rate, and the addition of a third substance, the catalyst, changes that rate, three possibilities exist. Addition compounds may be formed made up of the two substances and of the two substances plus catalyst. The combination of catalyst and either one of the reacting substances obviously is also possible but will not be considered at this point. The formations of these two addition compounds and their decompositions evidently represent simultaneous reactions. If the velocity of the reaction involving the catalyst is less than that of the other reaction (actually the sums of the velocities of the formation and decomposition of the addition compound are meant) then probably only a small part, if any, of the reaction will follow that course and the velocity will be practically unchanged. The velocities of the simultaneous reactions may also be equal. In the third case, the reaction with the catalyst is the more rapid and will be the reaction measured. This is generally true with the substances recognized as catalysts. If a substance does not increase the velocity, it apparently does not take part in the reaction and is not called a catalyst. Negative catalysis may often be due to the course of the reaction being changed or a different set of products formed because of the presence of the catalyst. In reactions I (a) and (b), a small amount of water would be able to take part in the reaction with considerably larger amounts of the other substances. Again, considering reactions II (a) and (b), platinic chloride catalyzes the reaction between ammonia, hydrogen chloride, and ammonium chloride; from reactions II (a) and (c), hydrogen chloride may act as the catalyst; and from reactions II (a) and (d), ammonia may act as the catalyst. Similar reasoning may be used with reactions I. The addition of the catalyst increases the number of possible sets of equilibria. At the same time, due to the nature and properties of the catalytic substance, one of the possible reactions will be favored over the others as a rule and the reaction observed takes that course. While these relations are true, it is evident that catalytic reactions follow the same rules as chemical reactions in general, and in fact are simpler in the sense that the chemical composition of one substance is unchanged. That this substance takes part in the reaction is evident from the fact that the velocity of the reaction is changed. The primary formation of an addition compound, however, holds for a catalytic reaction in the same way as for any other reaction. Some of the evidence for this with enzymes, a special group of catalysts, will be given later.

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[9] **Cf. G. Bredig, *Ergebnisse der Physiologie 1,* 134 (1902); J. Stieglitz, *Proc. Congr. of Arts and Sciences,* St. Louis, 1904, Vol. IV, p. 276; and others.**

In fact the evidence with regard to the formation of intermediate compounds in enzyme actions is one of the supports of the theory of addition compound formation. Logically, the evidence is presented in a reverse order here, but the general view, that enzyme reactions form one group of chemical reactions in general and follow the same regularities and laws, in a measure justifies this method of presentation.

To sum up some of these relations, chemical reactions between two or more substances take place with the primary formation of addition compounds which then react further. The velocity observed in any given case depends upon the velocities of the separate reactions. In a catalytic action, the chemical composition of one of the reacting substances or components, perhaps in the sense that component is used in phase rule discussions, is the same before and after the reaction, but the velocity of the reaction observed is different. Not every substance which is unchanged in composition but forms addition compounds and takes part in a reaction need change the observed velocity.

It was stated that the velocity of a reaction may be changed by various amounts of added substance, ranging from a change in solvent to the addition of minute quantities of catalysts, such as acid in sucrose hydrolysis, etc. The latter have always been included under catalysts, and it is a question as to how far the term catalyst should go. Catalysts are often defined as substances present in small amounts and having the properties indicated. The definition of amount offers a real difficulty, however. There seems to be no reason to limit the definition of catalyst in this way. As stated already a substance may be present in any quantity and act as catalyst. If the velocity of the reaction is changed by the change in solvent, it appears as though the solvent took an active part in some way in the reaction. From the theory used here, it would be involved directly in the formation of the addition compound. Although this point is of no direct importance in connection with the problems to be taken up here, it is mentioned for the sake of completeness. The possible change in solvent by the addition of comparatively small amounts of foreign substances which results in a change of velocity in the reaction taking place in the solvent is of importance however and will be referred to again in later chapters.

The presence of a catalyst is not supposed to change the equilibrium of a chemical reaction according to the views used heretofore in most discussions. From time to time, statements have appeared in the literature taking exception to this view without, however, going to the root of the question and attempting a classification and description of catalytic actions which would eliminate such apparent contradictions. Thus, G. Bredig[10] showed that a change in the vapor pressure of a catalyst necessitates a difference in the work required to remove the catalyst from the reaction mixture. Only as long as this work was the same under the same conditions before and after the reaction, did the equilibrium remain unchanged. If the catalyst was present in large excess it acted as solvent. A change in the nature of the solvent changed the equilibrium, and only in dilute solution would the equilibrium remain the same. E. Abel[11] stated, assuming the formation of intermediate products with the catalyst, that if the catalyst was in a different chemical or physical state at the end of the reaction from what it was at the beginning, it had given up or received energy, and that consequently a change in the equilibrium was conceivable. J. Stieglitz[12] pointed out clearly the conditions under which the equilibrium of a catalyzed reaction would be changed. W. J. Jones and A. Lapworth[13] found experimental evidence for the change in the equilibrium between ethyl alcohol, acetic acid, ethyl acetate, and water, by the addition of the catalyst hydrogen chloride. M. A. Rosanoff[14] also spoke of the possibility of a catalyst influencing the equilibrium, and that it did not do so only when the molecular state of the reagents was not affected by the catalyst. A number of other chemists may be quoted in the same sense. Recently W. D. Bancroft[15] reviewed certain phases of catalytic reactions.

If a catalytic reaction is defined as a reaction in which one of the products is identical in chemical composition with one of the original substances involved in the reaction, the view which is used here, then it may be possible to arrive at definite general conclusions. No further limitations are introduced in the definition. In the general formulation of a chemical reaction:

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[10] *Ergebnisse der Physiologie 1,* 139 (1902).

[11] *Z. Elektrochem. 13,* 555 (1907).

[12] *Am. Chem. J. 39,* 56 (1908).

[13] *J. Chem Soc. 99,* 917 (1911).

$$V_1\alpha_1 + V_2\alpha_2 + \ldots n_1A_1 + n_2A_2 + \ldots\ldots = V_1^1\alpha_1^1 + V_2^1\alpha_2^1 + \ldots\ldots$$
$$n_1^1A_1^1 + n_2^1A_2^1 + \ldots\ldots$$

in which α_1, α_2, ... $\alpha_1^1\alpha_2^1$ .. represent the molecular species in the solid state taking part in the reaction, A_1, A_2......A_1^1, A_2^1.... the molecular species either as gas or in solution, and V_1, V_2 ... n_1, n_2, ... V_1^1, V_2^1,...n_1^1, n_2^1, ... the corresponding molecular species formed in the reaction, then the definition of catalytic action advanced requires only that one of the molecular species α_1^1, α_2^1, ...A_1^1, A_2^1, ... is identical in composition with α_1, α_2 ...A_1, A_2, ... The definition does not, *a priori*, state anything concerning the velocity of the reaction. Since one of the substances appears as a product of the reaction, obviously it may go through the cycle of the reaction again with fresh initial material. This substance is the catalyst, and therefore a small amount of this substance may react with a large amount of the other substances. This phenomenon has always been taken to be one of the most characteristic properties of a catalyst. With regard to the possibility of the reaction taking place in the absence of the catalytic substance, if every one of the substances at the beginning and at the end of the reaction is present in the pure state, or possesses the same physical and chemical properties, then the equilibrium constant derived from the law of mass action would be independent of the catalytic substances, the equilibrium would be the same whether the catalyst were present or not, and reaction would proceed in the presence or absence of catalyst, although the rates in the different cases may well be different. Furthermore, as stated before, it is impossible to predict the effect of the catalyst; it might increase the rate of the reaction, it might decrease it, or it is possible that no effect at all would be noticeable upon the rate of the reaction. The so-called "negative catalysis" (cf. Mellor,[16]) is then simply a special case of catalysis in general, the catalyst here retarding the reaction instead of accelerating it.

In practical work, the substances taking part in a reaction are hardly ever isolated in a pure state, so that a development of the ideal case just presented will be necessary. If, in the general formulation of a chemical reaction given above, the concentrations of the substances A_1, A_2, ... A_1^1, A_2^1, ... in the free state before and after the reaction are denoted by C_1, C_2, ... C_1^1, C_2^1, ... and their concentrations at equilibrium by c_1^1, c_2^1, ..., then it may readily be shown that the change in the free energy (A) of the reaction is given by the equation

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[16] J. W. Mellor, "Chemical Statics and Dynamics," 1909, p. 371.

$$A = R\ T \log_e \frac{C_1^{n_1}\ C_2^{n_2}\ldots.}{C_1^{1n_1^1}\ C_2^{1n_2^1}\ \ldots} - R\ T \log_e \frac{c_1^{n_1}\ c_2^{n_2}\ldots.}{c_1^{1n_1^1}\ c_2^{1n_2^1}\ \ldots}$$

(This equation is developed with the aid of the conception of the equilibrium box (van't Hoff) into which the reacting substances in dilute solution or the gaseous state are introduced through suitable semi-permeable membranes, in which the reaction proceeds, and from which the products are removed through suitable semi-permeable membranes, all isothermally and reversibly, the work done in the different steps being calculated by the aid of the gas laws.) The fraction of the second term of the right side of the equation is the equilibrium constant K. If the states of the substances are such that each exists independently before and after the reaction, then, even if $c_1^{n_1} = c_1^{1n_1^1}$ as necessary for a catalytic change, the work done will be the same whether the catalyst is present or absent, and the equilibrium will be unchanged. If, however, work is done in introducing or removing the catalyst, some sort of chemical compound is formed between two or more of the molecular species, whether this be called chemical combination, solution, adsorption, physical change, etc., and then the terms involving the concentrations will not be the same as before. Thus the concentration term $c_1^{n_1}$ may denote a complex containing the catalyst, while in the denominator the catalyst may be represented in a different complex. It may be said, therefore, that in the chemical changes as ordinarily observed, if a catalyst is involved in the reaction, as long as the substances are not all present in the pure state or possessing the same properties before and after the reaction, there may well be a difference in the change in free energy of the reaction in the absence and presence of the catalyst and that the equilibrium will be changed correspondingly. That such changes have not been observed more frequently than has actually been the case is doubtless due to the small changes in the equilibria which have resulted by the addition of the catalyst.

IV.—Chemical Reactions Catalyzed by Enzymes

The chemical reactions whose velocities are increased by enzyme preparations include a number of comparatively simple reactions as well as many complex ones. That is to say, the transformations which take place may involve simple chemical changes which are more or less well known and which can be followed satisfactorily. It is with these reactions that it would appear that further insight into the enzyme problem will be gained. It is true that some things can be done and some advances made even by means of enzymic reactions with such complex substances as proteins and starches. In these, however, the two-fold difficulty of dealing on the one hand with substances which, while chemically characterized as belonging to certain types, are unknown even so far as their chemical composition is concerned, and on the other hand using preparations to accelerate reactions which are chemically practically entirely uncharacterized, makes it appear as if more is to be hoped for at the present time with the simpler reactions.

In this chapter, some of the reactions which are to be included in enzyme actions will be considered entirely aside from the enzyme part of the problem. It is not necessary to enter into all of the reactions, even of the simpler ones, which might be included. Of the hydrolytic reactions, the hydrolysis of sucrose and of esters will be considered somewhat in detail, and in addition oxidation reactions will be taken up.

These two hydrolysis reactions have been chosen because they have been studied very extensively from the chemical point of view and beause the enzyme preparations which accelerate the reactions have also been studied to a considerable extent. It should be obvious that, with the enzyme as the unknown factor, as much information as possible with regard to the chemical reaction itself should be at hand in order to elucidate the action and nature of the unknown factor, the enzyme. In the rest of this chapter enzymes will be considered only incidentally. Attention will be focused on the chemical reactions as such.

The empirical equation representing the hydrolysis of sucrose may be written as follows:

$$\underset{\text{sucrose}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{glucose}}{C_6H_{12}O_6} + \underset{\text{fructose}}{C_6H_{12}O_6} \qquad (1)$$

The possible action of other substances present in solution is not given. This equation represents, however, only the barest outline of the changes occurring. Questions of isomerism must also be considered. A more complete statement of the changes taking place may be shown in the following outline:

$$\text{Sucrose} + \text{Water} \begin{array}{l} \nearrow \ \alpha\text{-glucose} + \beta\text{-glucose} \\ \\ \searrow \ \alpha\text{-fructose} + \beta\text{-fructose} \end{array} \qquad (2)$$

Equations (2) may be written in such a way as to indicate the structural formulas of the various substances involved. This is done in equations (3) on page 45.

α-Fructose has not been isolated as yet in the pure state. The structural formulas to be assigned to the two fructoses are not quite definite, but it is certain that there is an α- and β-form, and that an equilibrium exists in solution between these forms.

The most recent work[1] on the structures of these compounds has shown that the β-fructose can exist in two forms, either as the ethylene oxide or the butylene oxide structures. Sucrose itself is in all probability a compound consisting of a butylene oxide aldose (d-glucose radical) and an ethylene oxide ketose (d-fructose radical). It is not a simple glucoside or fructoside. The general hydrolysis can now be represented schematically as follows:

$$\begin{array}{lccccc} \text{Sucrose} \ [\rightarrow] & \alpha\text{-glucose} & + & \alpha\text{-fructose} & [\rightleftarrows] & \alpha\text{-fructose} \qquad (4) \\ & \uparrow\downarrow & & \uparrow\downarrow & & \uparrow\downarrow \\ & \beta\text{-glucose} & & \beta\text{-fructose} & [\rightleftarrows] & \beta\text{-fructose} \\ & \text{(butylene oxide} & & \text{(ethylene oxide} & & \text{(butylene oxide} \\ & \text{forms)} & & \text{forms)} & & \text{forms)} \\ & \text{(A)} & & \text{(B)} & & \text{(C)} \end{array}$$

The arrows in brackets represent changes involving structure; the others indicate stereochemical interconversions. In the hydrolysis of sucrose, the final product consists of A (butylene oxide forms of α- and β-glucose) and C (butylene oxide forms of α- and β-fructose) and probably a small proportion of B (ethylene oxide forms of α- and β-fructose).

Equations (2), (3), and (4) represent the hydrolysis of sucrose

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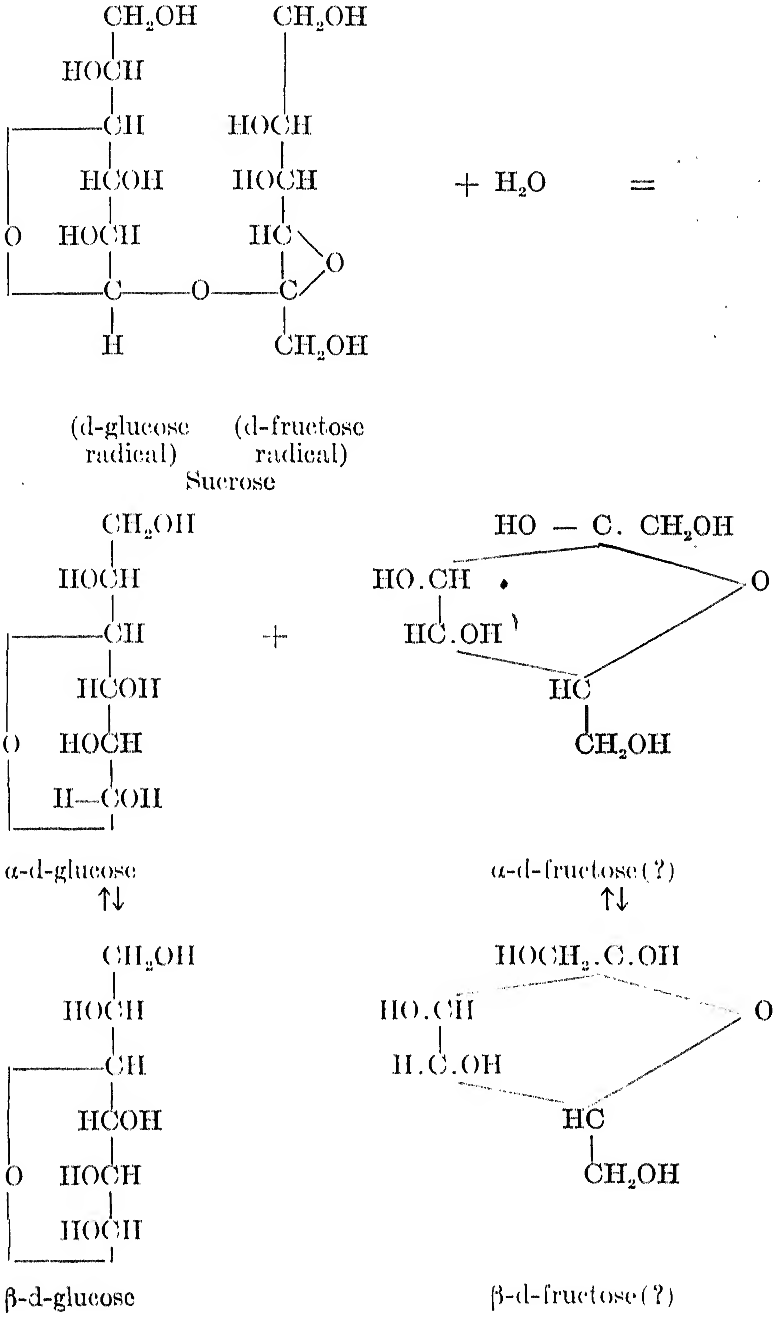
[1] W. N. Haworth and J. Law, *J. Chem. Soc. 109,* 1314 (1916); E. F. Armstrong, "The Simple Carbohydrates and the Glucosides," Monographs on Biochemistry, III Edition, 1919, p. 100.


+ H2O =
(d-glucose radical)
(d-fructose radical)
Sucrose
+
α-d-glucose
α-d-fructose(?)
β-d-glucose
β-d-fructose(?)

(3)

as it is assumed at the present time to occur. The probable primary or intermediate compound between sucrose and water which precedes the formation of the hexoses is, however, not indicated in the formulations. The first step given in the equations is the reaction between sucrose and water to form α-glucose and α-fructose. This reaction, taking place as a rule in aqueous solution, is practically irreversible under the conditions which have been used. The α-forms of glucose and fructose react further going over into the β-forms This change is known as mutarotation and does not go to completion ordinarily. A mixture of the α- and β-forms is present in any given case. The mechanism of the change from the α- to the β-form or vice versa in all probability depends upon the formation with water of an intermediate compound, probably a monohydrate.[2]

As stated earlier in this chapter, it is advisable to know as much as possible of the chemical and physical properties of the substances whose chemical changes are being followed kinetically. Before going on to the velocity of the indicated reaction, a few of the properties of the substances may be referred to. The rotation of the plane of polarized light of these compounds in solution is perhaps the property of which use is most commonly made. The spatial arrangements of the atoms and groups is indicated in the formulas of equations (3). The hexoses are both the dextro forms. The changes in rotation in the various stages of the reaction are marked enough to permit of the determinations of the changes by these means. The specific rotation of sucrose in solution is $[\alpha]_D^{20°} = +66.7°$.[3] For α-d-glucose the best value was found to be 111.2°, for β-d-glucose 17.5°. In aqueous solution at equilibrium the value is 52.5°; or the amount of α-form present is 37.4% and of β-form 62.6%. For β-fructose the specific rotation is 130.8° between 0.15 and 37°, at equilibrium the rotation for d-fructose is —88° at 25° C.[4] Since α-fructose is not known in a pure state, its value cannot be given.

Another property which may be of importance in some cases in following these changes in solution, is the viscosity of the solution. For solutions which are concentrated to any considerable degree, since kinetic actions are involved, it would appear to be advisable to refer all changes to solutions possessing the same fluidity or viscosity. This is true also for solutions containing foreign added substances.

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[2] T. M. Lowry, *J. Chem. Soc. 75,* 213 (1899); *83,* 1314 (1903); *85,* 1551 (1904). E. F. Armstrong, *J. Chem. Soc. 83,* 1305 (1903).

[3] Cf. J. E. Mackenzie, "The Sugars and Their Simple Derivatives," 1914, pp. 28-9.

[4] J. M. Nelson and F. M. Beegle, *Jour. Amer. Chem. Soc. 41,* 559 (1919); cf. also C. S. Hudson, *Ibid. 31,* 655 (1909); C. S. Hudson and E. Yanovsky, *Ibid. 39,* 1013 (1917); W. C. Vosburgh, *Ibid. 42,* 1696 (1920).

As for chemical properties or reactions, the one which is used frequently is the reducing power of the hexoses formed on alkaline cupric salt solutions such as Fehling solution and others. Sucrose does not react under certain definite conditions, so that the amount of change can be followed by the amount of cuprous oxide formed. The hexoses are oxidized in the reaction, and it is obvious that it is necessary to adhere strictly to fixed conditions which have been standardized with known solutions to obtain comparable results, because of the manifold possibilities of oxidation of the hexoses. If this is done, accurate results may be obtained,[5] and at times this method possesses advantages over other methods of following the changes. The property of the reducing actions of the hexoses may be used with other reagents,[6] but the general principles of the reactions remain the same.

The hydrolysis of sucrose has so far been assumed to take place in aqueous solution without the addition of any other substance. At moderate temperatures this reaction is extremely slow. Acids accelerate the reaction and as a first approximation, with monobasic acids, the greater the strength of the acid as measured by its electrolytic dissociation or ionization in solution, the greater its catalytic action. The velocity of the reaction has been measured a great number of times, and the kinetic equations outlined in Chapter II applied. Their application appears simple at first sight, but closer study has revealed a number of complicating factors. In solutions so dilute that the active mass (or concentration) of the solvent does not change appreciably during the measurements, the velocity of the reaction might be expected to follow the monomolecular law, that is, the amount of change being proportional to the concentration of sucrose present at any instant. The two methods of measuring the change which have been used are the change in rotation of polarized light and the reduction of alkaline copper solution. The reaction in which the α-forms of the hexoses are transformed to the β-forms is apparently involved in the measurements, but it was found that equilibrium between the different forms is reached fairly rapidly in the presence of acids.

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[5] Cf. H. C. Sherman, E. C. Kendall and E. D. Clark, *Jour. Amer. Chem. Soc.* *32*, 1073 (1910), for comparison of different methods of determining reducing actions of sugars.

[6] Such as picrate. Cf. R. C. Lewis and S. R. Benedict, *Proc. Soc. Exp. Biol. and Med.* *11*, 57 (1913-14); *J. Biol. Chem.* *20*, 61 (1915); S. R. Benedict and E. Osterberg, *J. Biol. Chem.* *34*, 195 (1918); R. Okey, *J. Biol. Chem.* *38*, 33 (1919); W. M. Dehn and F. A. Hartman, *Jour. Amer. Chem. Soc.* *36*, 403 (1914); K. G. Falk and H. M. Noyes, *J. Biol. Chem.* *42*, 109 (1920).

The proportionality between the hydrogen ion concentration as determined by the conductivity method and the velocity of reaction was found to hold quite satisfactorily in dilute solutions of acids as indicated in the second chapter. However, in the more concentrated solutions of the acids, or in the presence of added salts, this proportionality did not hold. That is to say, the values of k of the velocity equations were no longer constant for a series of determinations with the more concentrated)acid, or the ratio of the constant of the velocity equation to the hydrogen ion concentration varied with different concentrations of the acid. In order to account for these variations, modifications of the original theory were proposed. A brief review of some of the various theories suggested is of importance in this connection.

In considering the catalytic actions of acids on the hydrolysis of sucrose, three sets of explanations or theories to account for the reactions have been proposed and are used at the present time. In the first place, the action has been considered as due entirely to the hydrogen ions present in solution; second, the Dual Theory of catalysis assumes the action to be due both to the hydrogen ions and to the unionized molecules; third, the addition theory of chemical reactions assumes the intermediate or primary formation of an addition compound, with the acid molecule, and considers that the action of the solvent is involved as one of the main factors, while the ionization is secondary.

The three points of view will now be considered in more detail since they apply to all of the reactions to be considered. In the first, the action is stated to be due entirely to the hydrogen ion. The velocity constants of the reaction with acids was found to be practically proportional to the equivalent conductivity of the acid in dilute solutions. Since this equivalent conductivity is due mainly to the concentration of the hydrogen ion, the catalytic action was taken to be due to the hydrogen ion. Other reactions whose velocities were accelerated by acids were found also to parallel very closely these changes and were used as additional evidence. This relation was developed between 1880 and 1890 and received strong support from the theory of electrolytic dissociation of Arrhenius.

As data on this reaction accumulated, especially with more concentrated solutions, the simple explanation of the action of hydrogen ions was found to be insufficient. For example, nitric acid, 0.5 N, containing, from conductivity determinations, 4.64 as many hydrogen ions as a 0.1 N solution, hydrolyzed a sucrose solution 6.07 times as fast.[7] While this and similar apparently "abnormal" actions of acids may be explained away in various ways, greater difficulties are encountered in the reactions where neutral salts are added, which in place of decreasing the actions because of repressing the ionization of the acid frequently increased them. These phenomena were developed for a number of reactions from 1907 on by S. F. Acree, G. Senter, S. A. Arrhenius, G. Bredig and H. C. S. Snethlage, H. Goldschmidt, A. Lapworth, H. S. Taylor, H. M. Dawson, and others. The explanation advanced and called by Dawson the Dual Theory of Catalysis,[8] takes the view that in addition to the catalytic action of the hydrogen ion, the unionized acid molecule exerts a catalytic action. For a number of reactions and acids the catalytic action of the unionized acid molecule, denoted by k_m, and the catalytic action of the hydrogen ion, denoted by k_h were determined using the gram-equivalent as the unit of mass.[9] The ratios of these values give the comparative effect of the catalytic actions of acid molecule and hydrogen ion. The strongest acids as measured by the ionization gave the largest values for the ratios, while the weak acids gave the smallest values. The values of the ratios ranged from about 2 for acids such as hydrochloric acid to less than 0.01 for very weak acids. For any one acid the ratio varied also with the reaction being catalyzed. This ratio, at first assumed to be characteristic for a given acid, was later found to vary in this way. If intermediate compounds with the catalyst are formed, a reason for this variation is apparent.

The dual theory of catalysis and the catalytic actions of unionized molecules leads to the third theory of the actions. The presence of hydrogen ions, or the phenomenon of electrolytic dissociation, is ascribed to certain properties of the solvent as in all the theoretical views. In the reaction catalyzed by the acid, an addition compound is assumed to be formed by the acid, sucrose, and water of the solvent, this primary addition compound then being able to react in several different ways, for instance, to form sucrose and acid; glucose, fructose, and acid, etc., as outlined in principle in Chapter III in discussing the general theory of chemical reactions. The composition of the intermediate compound qualitatively may be indicated; quantitatively it is unknown at present. The question of hydrogen ions naturally arises. This is not considered to be the predominating factor in this theory. The complex addition compound may ionize so that it is apparently an addition compound with the hydrogen ion, not with the acid. The ionization is considered as a secondary reaction which in itself plays no direct part but is only a physical indication that the solvent has brought about a change in the acid molecule, just as the chemical action of the sucrose hydrolysis is similar evidence of a parallel nature of the chemical change in the acid molecule brought about by the solvent.

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[7] Cf. J. W. Mellor, "Chemical Statics and Dynamics," (1909), p. 280.

[8] Cf. W. C. McC. Lewis, "A System of Physical Chemistry," Vol. 1, p. 423 (1918).

[9] A summary of these values was given by H. S. Taylor, *Z. Elektrochem.* **20,** 202 (1914).

It may be noted in this connection that the recent work on the theory of electrolytic dissociation is tending to modify most profoundly some of the foundations of this theory. A number of the most active workers in this field,[10] the most recent being A. A. Noyes,[11] have published papers in which the views are put forward that for highly ionized or strong electrolytes, electrolytic dissociation was complete, and that the observed and calculated values obtained for the percentages of ionization show deviations from the values for complete ionization because of secondary relations. If this view should prove to be correct and be accepted, the first two theories of the chemical reactions outlined here would require modification in that the chemical reactions can not be used as evidence in connection with or in paralleling the percentages of ionization.

The third view presented above cannot at present be formulated as precisely as the two earlier theories, partly because of the more complex nature of the reactions involving the intermediate compound. It appears, however, to be more flexible and of more general applicability.

Which of these views will be accepted ultimately cannot be foretold at present. They are presented in order to show the status of the theoretical side of the problem of the catalysis of sucrose by acids. Since this monograph does not include a general treatment of catalytic reactions, the detailed evidence which has been accumulated will not be presented, nor will a critical summary of the experimental work bearing on the question be attempted. For this the reader is referred to textbooks and other suitable summaries of the work on sugars and the studies on catalysis.

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[10] W. Sutherland, *Phil. Mag. (6) 14,* 3 (1907); S. R. Milner, *Phil. Mag. 35,* 214, 354 (1918); J. C. Ghosh, *J. Chem. Soc. 113,* 449, 627 (1918); N. Bjerrum, *Z. Elektrochem. 24,* 321 (1918).

[11] A. A. Noyes and D. MacInnes, *Proc. Nat. Acad. Sci. 6,* 18 (1920); *Jour. Amer. Chem. Soc. 42,* 239 (1920).

For the objects in view here, it will only be necessary to emphasize a few points in connection with the catalytic hydrolysis of sucrose. Increase in concentration of acid, whether measured by the increase in hydrogen ion concentration stated as values of pH,[12] or as concentration of acid in equivalents or mols, or in any other way, increases the rate of hydrolysis, not quantitatively, except possibly for the very dilute solutions, but in a very rough way, proportionately, or perhaps better, in the same direction. In alkaline solution, or even neutral, the velocity of the reaction is almost nil. Increase in temperature increases the velocity two- or three-fold for every 10° rise, as with chemical reactions in general. The addition of neutral salts to an acid solution increases the rate of reaction, although if acid is not present, the reaction does not take place with neutral salts alone in solution.

The reactions involving the hydrolysis of esters follow in principle closely those involving the hydrolysis of sucrose. Some additional facts have been observed which complicate the reactions in some ways and help to explain them in other ways. In the first place, in the hydrolysis of esters, the reaction is catalyzed by bases as well as by acids. Assuming the hydrogen and hydroxyl ions to be the active catalysts, it was found that for equivalent concentrations, the hydroxyl ion exerted about 1,400 times as much action as the hydrogen ion. It is evident, consequently, that in going from an acid to an alkaline solution, a hydrogen ion concentration where the acid concentration is approximately 10^{-6} normal will be found for the solution where the catalytic action of hydrolysis (or saponification) of the ester is a minimum, increasing continuously from this point with increasing concentration of acid or of alkali. This was observed by Wijs[13] a number of years ago. In the study of the velocity of the reaction of ester hydrolysis, the formation of acid from the ester complicates the application of the velocity equations especially where alkali is used as the catalyst.

The same general relations were found with the ester hydrolysis reactions as with the sucrose hydrolysis reaction. With acids, for dilute solutions, the velocity was found to be directly proportional to the hydrogen ion concentration. (For bases, similarly, proportionality was found to the hydroxyl ion concentration as illustrated in

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[12] pH represents the negative exponent of 10 (or the negative value of the logarithm to the base 10) of the number representing the hydrogen ion concentration.

[13] J. J. A. Wijs, *Z. physik. Chem.* *11*, 492 (1893).

Chapter II.) For more concentrated solutions, the proportionality did not hold. Also, with a weak or slightly ionized acid, addition of a soluble neutral salt of the acid diminished the ionization of the acid and decreased the catalytic action of the acid proportionately; but if the acid was highly ionized, addition of the salt not only did not decrease the catalytic action of hydrolysis, but actually increased it.

The different explanations or theories proposed to account for these catalytic actions are exactly the same as those given in connection with the sucrose hydrolysis. The observation of a minimum action at a certain hydrogen ion concentration is explainable by any of the theories since ionization is not necessarily included in the explanations. Neutral salts influence the velocity of the reaction, and in the Dual Theory of Catalysis, values similar to those already indicated have been calculated for the relative actions of unionized acid molecules and hydrogen ions. These differed in some cases quite considerably from the values found for the same acids with the sucrose hydrolysis catalysts.

As for the third explanation advanced above, experimental evidence has been obtained showing the existence of ternary compounds of acid, alcohol, and catalyzing acid, or of binary compounds of ester and catalyzing acid. This question was gone into in some detail in other connections and reference will only be made here to these publications.[14] It need only be added that questions of electrolytic dissociation do not enter here any more than in the sucrose hydrolysis catalysis as a primary factor; that the original acids (or bases) may ionize, that the (intermediate) addition compound may ionize, but that these ionization phenomena are secondary (and perhaps incidental) to the reaction taking place. As stated frequently, they may be taken as physical evidence of the reacting medium or complex, being, so to speak, "active" or doing something, just as the chemical change is chemical evidence of the same thing.

The two reactions outlined indicate the present status of the view held with regard to the action of catalysts. Reactions such as the hydrolyses of urea to form ammonia and carbon dioxide, of peptides to form simpler peptides and aminoacids, of proteins, etc., might be taken up in this connection as of more direct interest in connection with enzyme problems. This will not be done, however, partly because the experimental evidence is much less in quantity with these and also does not bring out any new points of view, and partly because whatever evidence has been obtained points in the same direction, and bears out the explanations and theories already developed with the two reactions considered in some detail.

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[14] K. G. Falk and J. M. Nelson, *Jour. Amer. Chem. Soc.* *37*, 1732 (1915), for references to the experimental evidence regarding the existence of these compounds. Cf. G. Baume and G.-P. Pamfil, *J. Chim. Phys.* *12*, 260 (1914); J. Kendall and co-workers, *Jour. Amer. Chem. Soc.* *36*, 1222, 1722, 2498 (1914); *37*, 149 (1915).

The views may be summed up by stating that a completely satisfactory theory is not held, but it is apparent that much progress has been made. The chemical compositions and properties of the reacting substances in a number of the reactions are fairly well known, and knowledge with regard to the mechanisms is growing. The purely ionic point of view is being left behind, and more general theories involving unionized molecules, action of solvent, etc., are being developed. While the writer is undoubtedly biased, it appears to him that the general addition theory of chemical reactions, of which catalytic reactions form one group, offers the best ground for a general classification and explanation on the basis of related phenomena, for the observed facts.

Oxidation reactions appear to be somewhat more difficult to handle from the theoretical side than are hydrolysis reactions in connection with enzyme studies, possibly because of the apparently greater complexity of the reactions involved and also because of the smaller amount of careful experimental work done with them.

The three general types of oxidizing enzymes may be described briefly:

(1) Oxidases, which accelerate the oxidation of a number of different organic substances, such as alcohols, aldehydes, phenols, amines, etc.

(2) Peroxidases, which accelerate the oxidation of similar substances in the presence of hydrogen peroxide.

(3) Catalases, which accelerate the decomposition of hydrogen peroxide, evolving gaseous oxygen.

In considering oxidation reactions in general, reference may be made to Chapter I, where the theoretical views based upon the electron conception of valence were outlined. These views will not be used further here. The experimental work with the various oxidizing enzyme preparations has not as yet reached the stage where generalizations can be obtained from the enzymes from different sources and the various reactions which they catalyze, or specific substances whose oxidation reactions they catalyze. There is considerable variety in the different substances which may be oxidized. The time seems to be ripe for a careful and systematic study of these oxidation reactions and the enzymes which are connected with them. The application of the electron conception of valence or some analogous theory will unquestionably throw light upon and aid in systematizing the reactions involved. Until this has been done, however, the chemical nature of the substrates will not be considered in detail, only the general views on oxidation reactions being presented.

The reactions which are accelerated by oxidases depend upon the oxidation of a substrate without the addition of any other substance. The oxidation must involve, therefore, oxygen of the air or the reduction of some atom of the oxidase preparation. The former seems in every way to be the more likely assumption. This would mean then that the reactions are auto-oxidation reactions catalyzed by the enzyme preparation. A review of "Autoxidation of Organic Compounds" was published some years ago.[15] For the historical development of the subject and an enumeration of a great number of reactions reference may be made to the publications of C. Engler and J. Weissberg[16] and of J. W. Mellor.[17] The most probable course of these reactions indicates that molecular oxygen is added to the substance being oxidized, and that this "peroxide" may then decompose, part of the oxygen being given off readily and being able to bring about further oxidations. It is difficult to state the exact part taken by the oxidase in these reactions.

The peroxidase reactions are similar except that in place of the oxygen of the air taking part in the reaction, hydrogen peroxide is required. It would appear superficially that the peroxidase enzymes are not as active, if this very loose and unscientific term may be used, as the oxidase enzymes which accelerate reactions in which the molecular oxygen of the air is involved, while the former need hydrogen peroxide, which as a rule oxidizes substances more rapidly. The part played by the peroxidase is also not definite. That the reaction takes place in steps involving the formation of intermediate addition compounds appears to be unquestioned, but further than this, it appears to be impossible to go at present.

These relations show the present unsatisfactory state of the problem of the oxidizing enzymes and the possibilities in the way of systematizing the relations.

With catalase, the present state of the study is almost as unsatisfactory. The decomposition of hydrogen peroxide appears to be similar to the action of a number of colloidal metals in accelerating the same reaction, but aside from the probability of the formation of intermediate compounds of some type, nothing can be said with regard to the reactions.

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[15] K. G. Falk, *School of Mines Quarterly,* *29,* 15 (1907).

[16] "Kritische Studien über die Vorgänge der Autoxydation," (1904).

[17] "Chemical Statics and Dynamics," pp. 304-339.

Reducing enzymes have been described from time to time, especially in recent years. From the theoretical side, the reactions which are involved may be treated similarly, but it does not seem worth while to enter into this phase of the problem here until more is known of these reactions.

V.—Physical Properties Common to Enzyme Preparations

In the description of various preparations which are classed as enzyme preparations, it has been found that there are a number of properties which may be said to be common to all. That is to say, these different preparations from various sources have certain reactions or properties which are similar to a certain extent. These similarities were originally observed in a purely empirical manner, and gradually as a result of observing them, it became customary to define or describe an enzyme preparation as possessing the properties which had been found to be more or less common to those previously studied.

In this and the following chapters some of these properties will be discussed. It will be noted that in the headings of the chapters a distinction has been made between the physical and the chemical properties common to enzyme preparations. It is impossible to draw a definite line of demarcation between the two sets of properties. Any distinction or classification of this sort which is made is to a great extent arbitrary and dependent upon the personal point of view of the one making this distinction. This will appear more clearly in the following pages.

The first characteristic property of enzyme preparations which is met with in working with them is the colloidal property. Practically all enzymes exist as colloids. They do not dialyze, or dialyze extremely slowly through collodion or other suitable membranes. Advantage is taken of this property in separating the active enzymes as far as possible from accompanying inactive material which does dialyze, and also when certain enzyme preparations are dissolved in salt solution, for the purpose of purifying the enzyme material in subsequently removing the salt. The conclusion may be stated that although there is scarcely an enzyme preparation which has not been treated in this way, it is unfortunately true that the direct information with regard to the chemical nature of enzymes or of the conditions for their actions, which has been gained as the results of dialysis studies, is practically negligible.

Many substances of biological or biochemical origin do not dialyze through membranes and it might be considered that this colloidal property of enzyme preparations is due to the fact that they are obtained from such biological material and retain or possess their properties, while the actual enzymic action is in itself unconnected with the colloidal nature of the substance. This brings up at once a question which has been considered frequently, lately in some detail by W. M. Bayliss in his monograph on "The Nature of Enzyme Action,"[1] whether the predominating feature of enzyme action is to be taken to be the property of adsorption possessed by enzyme preparations largely because of their colloidal nature, or whether the reactions may be considered to be more chemical in character. Bayliss inclines to the view that adsorption is the prime factor involved and his views may be summarized briefly here, although reference must be made to his monograph for the detailed evidence.

Bayliss gives reasons for believing that the action of enzymes in general must be regarded as exerted by their surface, resulting in the formation of a colloidal adsorption compound with the substrate. He states that "By surface condensation the reacting constituents are brought into intimate contact and accelerated by mass-action. Whether chemical combination between enzyme and substrate occurs in any stage of the process is not yet decided. Direct experimental proof exists that enzymes act by their surfaces in liquids in which they are completely insoluble." In fact, Bayliss appears to have been the first to advocate the view[2] that the rate of change is a function of the degree of adsorption in the different stages of the reaction. The increased rate of reaction is considered to be due to the increase in active mass (concentration) owing to concentration on the surface. In order to account for the fact that certain substances are adsorbed by a certain surface, the chemical nature of the surface in question must be considered. This is specially true when considering specificities of enzyme action, markedly so with optical isomers, where in a partial analogy to the "lock-and-key" simile of Emil Fischer (which will be taken up again later) "it may be said that the chemical configuration of the surfaces of contact, or the molecular shape of the constituents of the surfaces, are potent factors in determining the possibility of intimate contact between them." He also points out that adsorption may be followed by chemical combination with the surface of the enzyme, although there is no evidence that it occurs. Following the adsorption of the substrate on the enzyme, an equilibrium is attained with the products of reaction from the substrate.

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[1] IV Edition, published by Longmans, Green and Co., 1919.

[2] W. M. Bayliss, *Biochem. J. 1,* 175 (1906).

The adsorption point of view as advocated by Bayliss and superficially stated here lays emphasis on the physical phenomenon of adsorption as the controlling factor in accelerating chemical change. At the same time, Bayliss considers the possibility of chemical reaction with the surface being the predominating factor, but prefers the former view, pointing out, however, that at the same time, the physical properties of a surface are conditioned by its chemical nature.

The writer prefers to look upon enzyme action as essentially chemical in character. The reactions would follow the general laws of chemical reactions, and if the mechanism outlined in Chapter III be accepted, the theory of intermediate compound formation, including catalytic reactions, should be applicable. The isolation and identification of intermediate compounds between substrate and enzyme has not been successful as yet, but at the same time it must be remembered that the nature of enzyme molecules is quite obscure. With regard to adsorption phenomena, it might be considered that the purely physical phenomenon takes place first and is followed by chemical reaction with the enzyme as catalyst. The enzyme preparation might also adsorb or be adsorbed by a substance which is not changed by it or whose velocity of transformation is not affected by it. At the same time, such views should include the possible, and even probable, relation of the chemical properties of the reacting constituents upon the formation of adsorption compounds. The point of view recently adopted by Langmuir and by Harkins in connection with the orientation of molecules of liquids on surfaces carried over to adsorption, which was given in the first chapter, seems to be specially relevant. W. D. Bancroft[3] has recently emphasized the same fact in an address on "Contact Catalysis" where he considers the increase in concentration in its effect on catalytic action, when reacting substances are adsorbed at the surface of a catalytic agent as relatively unimportant in most of the cases studied hitherto. He did not speak of enzymes as such, but only of catalytic actions in general.

E. F. Armstrong and T. P. Hilditch[4] arrived at analogous conclusions in a paper on "Catalysis on Solid Surfaces." Comparing the hydrogenation of organic substances (such as unsaturated fatty acids) to enzyme actions, they state: "In each case, the catalyst (enzyme or reduced nickel) unites primarily with the organic compound about to undergo change (hydrolyte or unsaturated glyceride), the complex so formed being decomposed by the other component of the interaction (water or hydrogen). In each case, moreover, action takes place entirely at the surface of minute particles and the activity of the catalyst depends entirely on the production of maximum surface and the avoidance of impurities likely to destroy or dirty this surface."

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[3] **Presidential address, 1920, American Electrochemical Society.**

[4] *Proc. Roy. Soc. London (A)* 96, 137, 322 (1919).

The following evidence points in the same direction:[5] Animal charcoal adsorbed scarcely a trace of glycine, but it adsorbed a little alanine and a comparatively large quantity of leucine. Similar variations were observed with polypeptides, even isomeric substances being adsorbed to very different extents by charcoal. The amounts adsorbed at various dilutions varied according to the adsorption law just as with enzymes. The adsorptive power of charcoal differed from that of enzymes in not being influenced by changes in the hydrogen or hydroxyl ion concentration of the solution. Since it was found that adsorption can occur with inactivated enzyme solutions, it was concluded that while adsorption of the substrate by the enzyme precedes fission, the latter process does not necessarily follow the former. Evidence for this is also shown by the behavior of glycyl-l-leucine, which, at 0°, is adsorbed by yeast extract, but not decomposed.

It seems simpler from the chemical point of view to lay the stress for enzyme actions, in the cases in which adsorption takes place as well as in those in which it is not so apparent, on the chemical actions between substrate and enzyme. With regard to the reaction velocity equation frequently taking the form of the adsorption equation, in view of the complexity of the reactions, especially with insoluble colloids, the application of kinetic equations in their simple form upon any basis appears to possess somewhat questionable validity. This question will be considered in greater detail in Chapter VIII.

Since the colloidal property is common to enzyme preparations, it appears to be advisable to discuss it somewhat further, especially since some recent work has added considerably to an understanding of the relations involved.

In recent years evidence has been accumulating that the chemical reactions which take place with colloids and in general on surfaces may be explained most satisfactorily upon purely chemical grounds. The reactions are analogous to reactions studied heretofore in homogeneous media. The laws and regularities involved have not been developed to as great an extent as with the latter, greatly because of the experimental difficulties encountered in studying a problem under new or changed conditions. The relations promise in time to be as clear and satisfactory as those for other branches of chemistry and in fact to be based upon the same underlying principles. These views have been developed gradually and by a number of different workers.

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[5] E. Abderhalden and A. Fodor, *Fermentforschung*, 2, 74, 225 (1917-18).

In order to show the type of work which underlies the present study of colloids, two investigations will be spoken of. The inorganic relations were outlined in part in a paper by H. T. Beans and H. E. Eastlack on "The Electrical Synthesis of Colloids,"[6] and studies on gelatine as an example of a biochemical colloid have been published by J. Loeb.[7] Beans and Eastlack studied especially the electrical metal colloid synthesis. They concluded that the formation of the colloid takes place in two steps, first a thermo-mechanical action of dispersion, followed by the formation of a colloidal complex between the highly dispersed metal and certain ions present in the medium. In a pure medium, containing no stabilizing ion, the dispersion behaves as an ordinary suspension and settles out rapidly. The interest in this paper lies in the indication for which experimental proof is given, that a colloid combines in certain ways with other substances or ions present and that the existence of the colloid is dependent upon the presence of these combinations. The experimental difficulties in such an investigation are enormous because of the possible action of minute quantities of foreign substances. More work along these lines is promised by the authors and will be eagerly awaited.

The work of J. Loeb on gelatine is perhaps more conclusive with regard to the results arrived at. He showed in a series of studies that gelatine, at its isoelectric point $[H^+] = 10^{-4.7}$ N or pH $= 4.7$, is, so to speak, at its transformation point. In more acid solutions it behaves as a salt in which the gelatine complex acts as the cation, in more alkaline solution it behaves as a salt in which the gelatine complex acts as the anion. A number of different properties such as the electrical conductivity, viscosity, solubility, etc., were followed over a wide range and these relations found to hold. At the isoelectric point the properties of the gelatine passed through a minimum (or maximum) in any of the series of measurements. At that point, the gelatine was present uncombined with acid or salt, and showed minimum solubility, etc. It is pointed out clearly that all of the reactions of gelatine can readily be accounted for on this basis, that it reacts chemically either as the positive or negative constituent of a salt depending upon whether the solution is on the acid or alkaline side of the isoelectric point. The chemical reactions of the gelatine complex in the different states (positive, neutral, or negative) might well be different, but the phenomena described appear to be sufficient to account for the observed facts.

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[6] *Jour. Amer. Chem. Soc.* 37, 2667 (1915).

[7] Series of papers in *J. Biol. Chem.*, 1917-18, and in *J. Gen. Physiol.*, 1919-20.

The generalizations found with gelatine apply evidently to all amphoteric colloids of the same nature. It will not be necessary to enter into these here. The fact may be emphasized that the chemical relations may be expected to underlie the phenomena in every case. The work of Sörensen and of L. J. Henderson and their co-workers, some of which has already been referred to, supplies important and valuable evidence in these directions.[8]

In the following chapter, this question of the effect of the reaction of the medium on the properties of the protein or other substance present will be discussed somewhat further, especially from the point of view of amphoteric electrolytes.

The discussion of colloids just presented appears to have no direct bearing on enzymes and enzyme problems. The reason for presenting the outlines of these recent careful investigations is that enzyme preparations being colloidal in property, any evidence connected with colloids will be of value in their study. This is true especially at the present time when the colloid studies are being pursued in a way so as to promise a satisfactory systemization of the subject in place of the theories and words used so frequently heretofore which often confused the questions involved. The advances in the scientific study of colloids will react rapidly upon the study of enzymes, and the properties of enzymes which may be dependent upon them will be put in a clearer and immeasurably more satisfactory light.

Some phenomena observed with enzymes will now be described, which are connected with the colloidal or similar properties just taken up, but which can not be readily accounted for at present.

The first interesting result is that described by Nelson and Griffin.[9] They found that a purified active sucrase preparation (of which more will be said in Chapter VII) was not affected in its activity whether or not the enzyme was adsorbed to a solid like charcoal, or to a colloid like saponin, serum, or egg albumin, or distributed uniformly throughout the solution of the substrate. Also it was found, and this is more significant, that the adsorbed sucrase can be displaced by a second colloid without any effect on its activity, and that it can be removed from an aqueous solution by adsorption to a solid and again brought into solution by a second colloid suspended uniformly throughout the solution.

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[8] For example, cf. S. P. L. Sörensen and others, *Compt. rend. trav. Lab. Carlsberg*, 1919, Vol. 12; also L. J. Henderson, E. J. Cohn, P. H. Cathcart, J. D. Wachman and W. O. Fenn, *J. Gen. Physiol. 1*, 459 (1919), for the results obtained with gluten.

[9] J. M. Nelson and E. G. Griffin, *Jour. Amer. Chem. Soc. 38*, 1109 (1916).

The second series of experiments which led to a similar conclusion were carried out by Northrop[10] in working with pepsin. Northrop found that the state of aggregation of the protein, whether in solution or not, and the viscosity of the medium, exerted no marked influence on the rate of digestion of the protein.

The third result to be given includes some work with a castor bean lipase preparation[11] where it was found that "whether the material was dissolved in salt solution or suspended in the aqueous solution appeared to be of small influence on its hydrolyzing action."

These investigations, dealing with three such different enzymes as sucrase, pepsin, and lipase, although it is true that all exert hydrolyzing action on their individual substrates, show what might be considered to be a more or less general physical property in that the state of aggregation exerts little or no influence upon the action of the enzyme. With regard to the significance of these actions in relation to the adsorption theory, they appear to indicate the great importance of the chemical nature of the enzyme in the actual reaction taking place.

Certain phenomena may be included here because related in some ways in physical treatment, although all enzyme preparations do not behave in the same way.

It has been found that many enzymes can be removed from solution by adsorption to other colloids. Thus alumina cream will remove practically all enzymes from their solutions,[12] and by suitable treatment these may be recovered from the aluminium hydroxide. The work of Nelson and Griffin on the removal of sucrase from solution has been referred to already. In speaking of solutions of these enzymes, a solution clear and homogeneous to the eye is meant, although the dissolved substances possess the properties of colloids, and might even, if the solution were sufficiently concentrated, appear non-homogeneous or even turbid.[13] The next step in this consideration involves the precipitation or coagulation of an enzyme preparation by the addition to the solution of a foreign substance, and also a similar precipitation by the removal from the solution of a substance apparently essential in holding the enzyme in solution.

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[10] J. H. Northrop, *J. Gen. Physiol. 1,* 607 (1919); W. E. Ringer, *Z. physiol. Chem. 95,* 195 (1915), obtained similar results for the action of pepsin on edestin.

[11] K. G. Falk, *Jour. Amer. Chem. Soc. 37,* 226 (1915).

[12] W. H. Welker and J. Marshall, *Jour. Amer. Chem. Soc. 35,* 822 (1913).

The addition of alcohol precipitates most of the enzyme preparations from their solutions. Different concentrations are required for different enzymes, and it has been found possible to use a fractional precipitation at different concentrations to aid in separating a part of the inactive accompanying material (cf. Chapter VII). This is illustrated by the procedure developed by Sherman and co-workers with amylases from different sources, and by others. The precipitate from the alcohol solution can be dried with ether and studied further. It is found then that some of the enzyme preparations retain their activity while others lose it by this treatment. Sucrase, amylase, papain, and others are not affected as a result of the alcohol precipitation and subsequent drying, while esterase, lipase,[14] and maltase,[15] are completely inactivated by the treatment. In all cases, as far as can be told, no physical or chemical change other than that indicated has taken place. The addition of acetone in place of alcohol produces the same result. The addition of certain salts parallels these actions. Precipitation with sodium fluoride for example inactivates the enzymes, esterase and lipase, at the same time precipitating them. Other salts have no or very slight actions, while others again increase the actions of the enzymes.[16] The reverse actions are also true in some cases. For example, the presence of a certain amount of inorganic salt, such as sodium chloride, is necessary in order to have amylase exert any action.[17] The removal of all of this salt inactivates the amylase, but addition again restores the activity. Various salts act differently, and it was found that at certain concentrations the alkali bromides exerted the most marked activating actions.[18]

These relations appear to be very confusing. They are given in this place in connection with the physical properties of enzyme preparations because the first apparent similarity of change in enzyme action by the addition of other substances is connected with the precipitation or coagulation of the enzyme preparations. A closer study of the changes reveals the fact that such precipitation may occur with unchanged activity, and that apparently reversible precipitation or coagulation may be accompanied by no other change in physical or chemical property which has been followed experimentally except change in enzyme activity.

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[13] A dilute aqueous solution of glycine appears clear and homogeneous to the eye, but a concentrated one may be cloudy or turbid.

[14] K. G. Falk, *Jour. Amer. Chem. Soc. 35*, 616 (1913).

[15] W. A. Davis, *Biochem. J. 10*, 31, 57 (1915).

[16] A satisfactory summary of such actions is given by H. Euler in his "Allgemeine Chemie der Enzyme." 1910, pp. 75-84.

[17] Cf. L. Petri, *Biochem. Z. 4*, 1 (1907); T. B. Osborne and G. F. Campbell, *Jour. Amer. Chem. Soc. 18*, 536 (1896); H. C. Sherman and co-workers, *Ibid.* series of papers, 1914-20.

[18] A. W. Thomas, *Jour. Amer. Chem. Soc. 39*, 1501 (1917).

This leads at once to an interesting deduction. If this is true, that no further change which has been detected experimentally heretofore results in a change in enzyme action, then it would follow that changes in enzyme action afford one of the most sensitive, if not the most sensitive, criterion of change taking place in biological or biochemical material. No other chemical method at present appears to be capable of detecting the physical or chemical changes which take place when, under simple treatments, enzyme actions are modified.

It may be thought that such changes in enzyme material are due possibly to changes in surface, but again the writer prefers to consider the changes as fundamentally chemical in character and the changes in surface, if such occur, to be secondary to and dependent upon the chemical changes.

It appears also that there is some connection between the colloidal properties of an enzyme preparation and the comparative stability of the enzyme. The colloidal properties are connected in a measure with a complex chemical structure or composition. That is to say, colloidal properties are found frequently with substances of large molecular weight. While, therefore, the colloidal property is found to be a characteristic of enzyme preparations, it is evidence of the fact that such preparations are derived from biological material, and also may be connected, through the complexity of the molecule, with the possibility of retaining the enzymic activity in the molecule, or some particular part of the molecule, by the influence of the rest of the molecule and its complexity. Evidence for this may be seen in the fact frequently observed that separation of the enzyme preparation from inactive material accompanying it in the natural state results in the enzyme becoming more sensitive to changes of inactivation. For example, O'Sullivan and Tompson[19] found that the conditions which resulted in inactivating a yeast sucrase solution at 50° in the absence of sucrose, in the presence of sucrose did not inactivate the sucrase at 60°

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[19] C. O'Sullivan and F. W. Tompson, *J. Chem. Soc.* *57,* 834 (1890).

and only inactivated it partially at 70°. Bayliss and Starling[20] showed that trypsin (obtained from pancreatic juice) was autolyzed much more slowly in the presence of proteins or peptones than in their absence. T. B. Osborne and G. F. Campbell[21] found that with amylase, the purer the preparation, the more sensitive was the enzyme to external conditions. The results of Sherman[22] point in the same direction. The following experiment of Bayliss[23] is also of interest in this connection. The presence of charcoal in a solution of trypsin preserved the enzyme to a considerable extent when heated to 60° for ten minutes; one-seventh less was destroyed than in the absence of charcoal, and charcoal is not a very effective adsorbent for trypsin.

Another series of properties which from one point of view may be considered to be physical in nature is the relation between acidity of medium and activity of enzyme. It has been found that the action of any one enzyme changes with the hydrogen ion concentration of the medium in which it acts, that with increasing acidity (starting with strongly alkaline solutions) the activity increases up to a certain hydrogen ion concentration and then decreases again with a still greater acidity, and that this maximum action or optimum hydrogen ion concentration is more or less sharp with different enzymes. A number of the values which have been found are given in the following table.

	pH
Sucrase, yeast[24]	3.7–5.2
Sucrase, yeast[25,26]	4.4–4.6
Sucrase, potato[27]	4 -5
Amylase, pancreatic[28]	7.
Amylase, malt[28]	4.4
Amylase, takadiastase[28]	4.8
Amylase, saliva[29] *	6.
Amylase, potato[27] *	6. -7.
Amylase, cabbage, carrot, white turnip[30] *	6.

*** These results refer to saccharogenic actions.**

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[20] W. M. Bayliss and E. H. Starling, *J. Physiol. 30*, 61 (1903).

[21] T. B. Osborne and G. F. Campbell, l. c.

[22] H. C. Sherman, l. c.

[23] W. M. Bayliss, *Proc. Roy. Soc. London, (B) 84*, 81 (1911).

References to Table.

[24] L. Michaelis and H. Davidsohn, *Biochem. Z. 35*, 386 (1911).

[25] S. P. L. Sörensen, *Biochem. Z. 21*, 131 (1909).

[26] H. A. Fales and J. M. Nelson, *Jour. Amer. Chem. Soc. 37*, 2769 (1915).

[27] G. McGuire and K. G. Falk, *J. Gen. Physiol. 2*, 215 (1920).

[28] H. C. Sherman, A. W. Thomas, and M. E. Baldwin, *Jour. Amer. Chem. Soc. 41*, 231 (1919).

[29] R. V. Norris, *Biochem. J. 7*, 26, 622 (1913).

[30] K. G. Falk, G. McGuire, and E. Blount, *J. Biol. Chem. 38*, 229 (1919).

	pH
Amylase, yellow turnip [30]	4. –7.
Pepsin (edestin, casein) [31]	1.4
Pepsin (egg albumin) [32]	1.4
Pepsin (caseinogen, 10-15 min.) [33]	1.8
Pepsin (egg albumin, ½-1 hr.) [25]	1.6
Pepsin (egg albumin, 12 hrs.) [25]	1.2
Trypsin, pancreatic (albumose) [34]	7.7
Trypsin, pancreatic (casein) [35]	8.3
Trypsin, pancreatic (casein) [36]	5.5–6.3
Trypsin, pancreatic (fibrin) [36]	7.5–8.3
Erepsin, intestinal (albumose) [37]	7.7
Protease, yeast (peptides) [38]	6.8–8.5
Protease, takadiastase (albumose) [39]	5.1
Pepsin, yeast (proteins) [40]	4.0–4.5
Trypsin, yeast (peptones) [40]	7.0
Erepsin, yeast (peptides) [40]	7.8
Pepsin, animal tissues (gelatine) [41]	3.0–3.5
Trypsin, animal tissues (peptone) [41]	7.8
Erepsin, animal tissues (glycylglycine) [41]	7.8
Papain (egg albumin, gelatine) [42]	5.0
Urease, soy bean [43] [44]	7.0
Maltase, yeast [45]	6.6
Maltase, takadiastase (47°) [46]	7.2
Maltase, takadiastase (35.5°) [46]	3.
Oxidase, vegetables [30]	7. –10
Peroxidase, vegetables [30]	7. –10
Catalase, vegetables [30]	7. –10
Catalase [25]	7.
Catalase, liver [34]	7.
Esterase, pancreatic [47]	8.3–9
Esterase, blood serum [47]	8.
Lipase, duodenal juice [48]	8.5
Lipase, gastric juice [48]	4. –5

[31] L. Michaelis and A. Mendelssohn, *Biochem. Z. 65*, 1 (1914).

[32] S. Okada, *Biochem. J. 10*, 126 (1916).

[33] L. Michaelis and H. Davidsohn, *Z. exp. Path. Therap. 8*, 398 (1910).

[34] L. Michaelis and H. Davidsohn, *Biochem. Z. 36*, 280 (1911).

[35] H. C. Sherman and D. E. Neun, *Jour. Amer. Chem. Soc. 38*, 2203 (1916); *40*, 1138 (1918).

[36] J. H. Long and M. Hull, *Jour. Amer. Chem. Soc. 39*, 1051 (1917).

[37] P. Rona and F. Arnheim, *Biochem. Z. 57*, 84 (1913).

[38] E. Abderhalden and A. Fodor, *Fermentforschung 1*, 533 (1916).

[39] S. Okada, *Biochem. J. 10*, 130 (1916).

[40] K. G. Dernby, *Biochem. Z. 81*, 109 (1917).

[41] K. G. Dernby, *J. Biol. Chem. 35*, 179 (1918).

[42] E. M. Frankel, *J. Biol. Chem. 31*, 201 (1917).

[43] E. K. Marshall, Jr., *J. Biol. Chem. 17*, 351 (1914).

[44] D. D. Van Slyke and G. Zacharias, *J. Biol. Chem. 19*, 181 (1914).

[45] L. Michaelis and P. Rona, *Biochem. Z. 57*, 70 (1913); *58*, 148 (1914).

[46] A. Compton, *Proc. Roy. Soc. London (B) 87*, 245 (1914).

[47] P. Rona and Z. Bien, *Biochem. Z. 64*, 13 (1914); *59*, 100 (1914).

[48] H. Davidsohn, *Biochem. Z. 49*, 249 (1913).

These results have been given in this form and in this connection because they have been found as a result of measurements generally classed as physical. Evidently, a great deal of accurate work has been done on determining the hydrogen ion concentrations at which various enzymes exert their maximum actions. It requires only slight consideration, however, to conclude that chemical phenomena of some form underlie these relations. As far as the accuracy and reliability of the determinations are concerned, it must always be remembered that when handling biochemical materials, changes are likely to occur on comparatively simple treatments. Irregularity of results, or apparently contradictory data, need not mean, therefore, that the work should be disregarded, but rather that closer study of the conditions under which the determinations were made should be attempted. The further consideration of these results, therefore, will be postponed to the next chapter, where emphasis is placed upon the chemical properties of enzymes.

VI.—Chemical Properties Common to Enzyme Preparations

In taking up chemical properties common to enzyme preparations, the difficulty which is met with right at the start is the fact that at the present time enzymes are not known as chemical individuals or definite entities and any discussion of the chemical properties of enzyme preparations would involve the necessarily uncertain factor that the property under discussion may have no connection with the enzyme action. This necessary limitation in the treatment must be borne in mind in what follows, and while it introduces a doubtful element into the conclusions, it will be shown that the common chemical properties which will be discussed are of general enough relevance to aid at the very least in indicating the directions which further studies may take, and possibly also may give results of immediate value.

The first property which should be taken up in a summary of this kind includes the chemical composition of the materials involved. The question may be asked whether analyses of a number of enzyme preparations show any results common to all. Enzyme preparations have been obtained in varying states of so-called purity, which means that different treatments have been used to remove enzymically inactive material.

A number of such preparations have been analyzed. The element at present of greatest interest in such preparations is nitrogen. In all the enzyme preparations which have been purified to a greater or less extent and then analyzed, nitrogen was found to be present. The percentage, however, varied greatly with different enzymes. A number of the results found for the solid preparations are given in the following table for some of the enzymes:

	% *N*
Amylase, malt [1]	15.1–15.3
Amylase, malt [2]	16.1
Amylase, pancreatic [3]	15.3

References to Table.

[1] H. C. Sherman and M. D. Schlesinger, *Jour. Amer. Chem. Soc. 37*, 643 (1915).
[2] T. B. Osborne, *Jour. Amer. Chem. Soc. 17*, 587 (1895).
[3] H. C. Sherman and M. D. Schlesinger, *Jour. Amer. Chem. Soc. 34*, 1104 (1912).

	% N
Sucrase, yeast [4]	1.3
Pepsin [5]	14.1–14.8
Pepsin [6]	14.6–14.9
Pepsin [7]	13.8
Esterase, castor beans [8]	16.2
Lipase, castor beans [8]	17.1
Lipase, soy beans [8]	15.5

A few words may be added in explanation of some of these results. Malt amylase (2) differs from malt amylase (1) in its mode of preparation in a longer time of dialysis. Malt amylase (1) showed considerably greater activity, and if dialyzed for as long a period of time as (2) gave an increased nitrogen content and smaller activity. The sucrase result is fairly characteristic for a number of the preparations obtained at different times. It has been claimed that sucrase solutions have been obtained which contained no nitrogen, but these results have not been corroborated. With regard to the pepsin preparations, successive purifications, by solution and reprecipitation, increased the percentage of total nitrogen somewhat, but decreased the content of amino nitrogen. The esterase and lipase results were obtained with inactive material from acetone treatment of the active enzymes.

These results, as well as others which might be quoted, show that every enzyme preparation which has been analyzed contains nitrogen. In the present state of knowledge of the combinations of phosphorus and the inorganic elements such as sodium, calcium, etc., these will be left out of detailed consideration, although it is possible, and even probable, that the state of combination of phosphorus, present unquestionably as an organic phosphate derivative in practically all of the preparations, will furnish valuable information when more is known of the internal structures of the protein, starch, and fat molecules. The relations of carbon and hydrogen are also difficult to treat in any general terms. Aside from the fact that they are present in proteins, fats, and carbohydrates, combined in the various ways common to organic compounds, not much can be said at this time.

To return to the nitrogen whose forms of combination have been used to such good purpose in studying biological material, the percentage present varies from a content of 1.3% in the sucrase of Nelson to 16–18% in the amylase of Sherman and in the castor bean lipase. The sucrase of Nelson prepared from yeast represents probably the most satisfactory, if not the purest, state and condition in which that enzyme has been obtained and studied carefully over a considerable period of time. The nitrogen present was combined as protein and, analyzed by the Van Slyke procedure,[9] was found to contain the usual nitrogenous constituents, but with an unusually large percentage (over 70%) of soluble monamino acids. The remainder of the sucrase preparation consisted of a carbohydrate phosphate complex. At the other extreme are the enzyme preparations which are made up of protein alone. In some, even the test for carbohydrate was negative. They showed on analysis the customary aminoacid distribution, differing in no distinctive way from similar analyses on inactive material from the same sources or in the make-up of proteins from other sources.[10]

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[4] J. M. Nelson and S. Born, *Jour. Amer. Chem. Soc. 36*, 393 (1914).
[5] C. A. Pekelharing, *Z. physiol. Chem. 35*, 8 (1902).
[6] T. B. Aldrich, *J. Biol. Chem. 23*, 339 (1915).
[7] L. Davis and H. M. Merker, *Jour. Amer. Chem. Soc. 41*, 221 (1919).
[8] K. G. Falk and K. Sugiura, *Jour. Amer. Chem. Soc. 38*, 921 (1916).

The question comes up here again as to whether the compositions as shown by such analyses do not represent merely the source of the enzyme material. The enzyme property might well be only a very small part of even the purest preparation which has been separated, and the analyses which have been given only show the general composition of the medium, if this term may be used, which carries or accompanies the enzyme.

Nitrogenous bodies, mainly of protein nature, form a part at least of all enzyme preparations. These proteins contain in the main a number of aminoacids linked in peptide combination. One of the first properties considered with aminoacids is their amphoteric character, ability of forming salts with either acids or bases. The properties of aminoacids, peptides, and more complex bodies, including proteins, when acid or alkali is added, the formation of salts and the resulting acidities of the mixtures or solutions, are of the greatest importance in the chemical study of these substances. This leads at once to the striking property common to all enzymes, that is, the change in activity with change in hydrogen ion concentration and the fact that for each enzyme there is a more or less sharply defined hydrogen ion concentration range over which the enzyme exerts its greatest action. A list of these hydrogen ion concentrations was given in the last chapter in connection with the common physical properties of enzyme preparations. In this chapter, an attempt will be made to take up some of the chemical relations which may underlie these optimum conditions and offer a possible explanation based upon the chemical structures.

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[9] D. D. Van Slyke, *J. Biol. Chem.* *10*, 15; *12*, 275 (1912).

[10] H. C. Sherman and A. O. Gettler, *Jour. Amer. Chem. Soc.* *35*, 1790 (1913); K. G. Falk and K. Sugiura, *Jour. Amer. Chem. Soc.* *37*, 217 (1915); K. G. Falk, *Jour. Amer. Chem. Soc.* *37*, 649 (1915).

First, however, it will be necessary to point out that the chemical reactions which are catalyzed by enzymes over limited ranges of acidity, are catalyzed by acids or alkalies over markedly different ranges, as indicated in Chapter IV. These enzyme actions, at the present time, must be ascribed to factors not included in the hydrogen or hydroxyl ions as such. For instance, yeast sucrase hydrolyzes sucrose best at pH 4.5, and the action is still marked at pH 3.5 and 6. In more alkaline solution the action is rapidly diminished, in more acid solution tests have shown that the sucrase is inactivated, but that here the hydrolytic action connected with the greater acidity comes into play and increases with increasing concentration of acid. With esters, aqueous solutions containing more acid or more alkali than a certain concentration in the neighborhood of pH 5 to 6, show continually increasing hydrolysis, differing in this from the esterases and lipases. The various proteases show limited hydrogen ion concentration optima, but in the absence of enzyme, the higher the acidity, the greater the hydrolytic action on protein material. These comparisons might be continued further but enough has been given to show the difference between acid and basic hydrolytic actions and enzymic hydrolytic actions.

It may appear as if these limited ranges of acidity in connection with certain chemical properties which have been found useful for certain purposes are unique. This is by no means the case, and the manner in which the preceding statement was made indicates at once another group of substances which possess certain chemical and physical properties of value to chemists over more or less limited ranges of hydrogen ion concentrations. These are the indicators. A brief outline of the theories which were developed for indicators and their uses may be of interest here, as a surprising parallelism to the present development of enzyme theories is apparent.

Before the development of the electrolytic dissociation theory of Arrhenius, a satisfactory systematization of the color changes of indicators was not possible. W. Ostwald[11] in 1894 attributed the different color of an indicator in acid or alkaline solution to the different colors of the ions and unionized molecules. If the indicator substance itself was an acid, the color in acid solution would be that of the unionized molecule, while in alkaline solution the color would be that of the negative ion. If the indicator substance was a base, the color in alkaline solution would be that of the unionized molecule, in acid solution that of the positive ion.

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[11] "Die wissenschaftlichen Grundlagen der analytischen Chemie," p. 104.

This theory was shown not to be general enough to include the observed phenomena, and was replaced by the "chemical" theory first suggested (for phenolphthalein) by Bernthsen, and developed by J. Stieglitz [12] who brought the chemical theory into harmony with Ostwald's theory of the sensitiveness of indicators, and by A. Hantzsch,[13] who showed the ionic theory of indicators to be highly improbable. The newer view considers every change in color of an organic substance to be due to an intramolecular rearrangement. Indicators form a special group in so far as the intramolecular rearrangements in their case are tautomeric in character and include, therefore, in most cases the shifting of a hydrogen atom in passing from one form to the other. The production of ions is secondary in the tautomeric changes, and if the ions are colored, it is because the unionized molecules from which they are derived are colored. The equilibrium between the tautomeric forms of a substance depends upon a variety of factors such as solvent, temperature, small amounts of certain added substances such as acids and bases, etc. To illustrate this, a few results obtained with ethyl acetoacetate may be quoted. The equilibrium between the tautomeric forms of this substance varies greatly in different solvents, the extreme values given by K. H. Meyer [14] being 0.4% enol form present in 3–5% aqueous solution at 0°, and 48% in hexane at 20°. A. Hantzsch [15] showed the important part played by solvents in affecting the equilibrium between the tautomeric forms of some indicators and, therefore, the color changes of indicators. The action of acid and of alkali on the equilibrium between tautomeric forms is well known. Similar actions take place with indicators in aqueous solution, one form predominating in the presence of acids, the other (tautomer) in the presence of bases. In practical titrations, the indicator substance is present in such small concentration that the color change which accompanies the transformation of one tautomer into the other is very marked with the relatively small amount of added substance necessary to produce it.[16] Other changes of conditions may be considered similarly for the indicators as a special class of tautomeric substances.[17] In general, it may be stated that the various factors which influence the equilibrium between tautomers also influence the equilibrium between the different tautomeric forms of indicators, and that the question of the electrolytic dissociation of the indicator substances does not enter into the theory of their color changes as assumed in the earlier theory, although it appears to be connected with one of the factors involving the sensitiveness. Some years ago, Wo. Ostwald[18] suggested the view that the colors of indicator substances were dependent upon their degrees of dispersion, and that acids and bases brought about color changes with them simply by altering the dispersion. This theory as an explanation of the phenomena has not found favor, however, and need not be considered further at present.

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[12] *Jour. Amer. Chem. Soc. 25,* 1112 (1903).

[13] *Ber. 39,* 1084 (1906) and numerous articles since. Cf. also among others, D. Vorländer, *Lieb. Ann. 320,* 116 (1902); *Ber. 36,* 1845 (1903).

[14] *Ber. 45,* 2843 (1912).

[15] *Z. Elektrochem. 20,* 480 (1914); *Ber. 48,* 158 (1915).

If in place of color change of indicators at a certain hydrogen ion concentration, chemical change due to enzyme is substituted, a number of striking similarities are apparent. Usefulness over a limited range of acidity may mean in terms of chemical configuration, that a certain definite chemical structure or relationship or combination between the atoms is present over that range and is modified or changed at different acidities. These changes are in the main reversible with indicators, very often irreversible with enzymes. Added substances, such as salts, etc., may modify the changes to small extents in some cases, to large extents in others. The development of the theories to account for the color changes are also similar to the present developments with enzymes; views involving colloidal properties, hydrogen ions, and, finally, the chemical theory, which has now been generally accepted and which has as its basis chemical structure as the reason for the definite property.

Since the optimum hydrogen ion concentration for enzyme action is more or less definite for any given case, attempts have been made to determine whether the activity was connected with or part of the unionized molecule or one of the ions. Experiments showing the direction of migration in solution in an electric field have answered this question for a number of the enzymes. Michaelis[19] gives the following summary of the results which have been obtained: with sucrase, the unionized molecule carries or contains the active enzyme; with pepsin, the cations are active, while with trypsin, erepsin, lipase, and maltase, the anions are the seats of the activities. Here again the possibility exists that the property which is being measured is a property of the medium carrying the enzyme. This was indicated with a preparation of purified pepsin which showed no direction of migration in an electric field until a protein (albumin or albumose) was added, when it migrated with the protein, assuming its (electrical) properties, or in other words, combining with it.[20]

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[16] In this connection cf. A. A. Noyes, *Jour. Amer. Chem. Soc.* 32, 815 (1910).

[17] For the action of neutral salts, cf. L. Rosenstein, *Jour. Amer. Chem. Soc.* 34, 1117 (1912).

[18] Z. Chem. Ind. Kolloide 10,

Another factor must be mentioned here in connection with the determination and the interpretation of the hydrogen ion concentrations for maximum enzyme actions. This point was emphasized clearly by Sörensen[21] in one of the first papers on the significance of the hydrogen ion concentration in enzyme studies. As a conclusion from the work of O'Sullivan and Tompson on sucrase and from some of his own work he stated: "An interrelation exists between the three factors: temperature, hydrogen ion concentration, and time. Optimum hydrogen ion concentration of an enzymic action varies, within fairly narrow limits, with the temperature and time of the experiment, and even the optimum temperature of such a reaction without doubt will vary with the time and the hydrogen ion concentration of the mixture, probably within narrow limits, depending upon the magnitude of the temperature coefficient of the velocity of inactivation of the enzyme. In indicating the optimum temperature and hydrogen ion concentration, the experimental conditions should also be given." O'Sullivan and Tompson found that for sucrase, as the reaction proceeded, the optimum hydrogen ion concentration tended to have a smaller value. Sörensen found that for catalase and for pepsin, the optimum condition tended toward a slight increase in acidity with longer times of acting. Compton[22] found that with maltase (from takadiastase) as the temperature was increased the hydrogen ion concentration for optimum action was changed. At 47° (duration of experiments, 16 hours) the value for the pH was found to be 7.2; at 35.5°, it was found to be 3. To state the relation in another way, the greater the acidity, the lower the optimum temperature and the more rapid the inactivation of the enzyme. In other words, optimum temperature and optimum hydrogen ion concentration are interrelated, and are specially important with enzymes, such as maltase, which are readily inactivated.

[20] C. A. Pekelharing and W. E. Ringer, *Z. physiol. Chem.* *75*, 282 (1911).
[21] S. P. L. Sörensen, *Biochem. Z.* *21*, 131 (1909).
[22] A. Compton, *Proc. Roy. Soc. London (B)* 88, 408 (1915).

Another factor which may also be mentioned is indicated in the table showing the hydrogen ion concentrations for maximum actions. For example, with pancreatic trypsin acting on casein, the optimum pH value was found to be 5.5–6.3; acting on fibrin, it was found to be 7.5–8.3.[23] The substrate evidently plays quite an important part in this reaction, and its possible influence must always be watched for in enzyme actions. This point will be taken up again.

The question must now be considered whether it is possible to go further at present with the general property of chemical structure for such unknown bodies as enzymes. Some space may be devoted to the fundamental properties of proteins and their simpler component parts and also of other biological materials.

For the moment, the discussion will be limited to the nitrogenous bodies. If an aminoacid is dissolved in water, the solution will possess a certain hydrogen ion concentration. This will vary to a certain extent with the concentration of the aminoacid, but only to a minor extent (and for the purpose in view this is negligible) with most of them. This hydrogen ion concentration is the isoelectric point of the aminoacid at which it is combined to a minimum extent with either acid or base. Adding acid or base in definite amounts to this solution, determining the resulting hydrogen ion concentrations, and plotting the amounts of acid and alkali against the hydrogen ion concentrations in terms of pH will give the titration curve of the substance. Each substance should have a more or less characteristic titration curve, depending upon the groupings present. This is true as well for peptides, peptones, proteins, etc., as for aminoacids. Each substance would be expected to have a definite isoelectric point, and following the developments of the preceding chapter, the properties of the substance would change in passing from the acid side of the isoelectric point to the alkaline. This method of treatment and the development of the use of titration curves in the study of the properties of proteins was developed especially by L. J. Henderson and his coworkers.

In the determination of the titration curves of proteins with a number of potential basic and acidic groups present, it is possible that in the addition of acid or alkali, combination with certain groupings takes place first and that in a sense there is a progressive neutralization of the basic or the acidic groups in the molecule. It is then conceivable that at some such point of partial neutralization, optimum conditions of enzyme action would be obtained, due either to the setting free chemically (possibly by rearrangement) of the active grouping, or a similar removal of an inhibiting grouping. This explanation is hypothetical, it is true, but it seems as if there must be some such chemical reason to account for the observed facts. The changes in colloidal properties which may occur are to be referred back to the differences in the chemical groups due to the added acid or alkali.

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[23] J. H. Long and M. Hull, *Jour. Amer. Chem. Soc.* **39**, 1051 (1917).

The question whether tautomerism or desmotropism plays a part here in a manner analogous to its action in indicators can only be referred to and will be taken up again in the following chapter.

The discussion of these last questions was limited to proteins. It is evident that other substances not protein in character but which may also be present in enzyme preparations might show the same relations. For example, the carbohydrate-phosphoric acid complexes which have been shown to be present in certain preparations, and possibly other phosphoric acid derivatives, might show these properties. In general, the same relations might be expected to hold and the influence of the hydrogen ion concentration taken to be fundamentally chemical in character and to modify the chemical state or the structure of the enzyme molecule or part of it.

In discussing titration curves and isoelectric points of proteins and other biological material, it must be remembered that any chemical treatment to which the protein is submitted may change its properties. Thus, repeated solution and reprecipitation, whether by the action of acid and base, or by salt solution and dialysis, or by alcohol or acetone, will unquestionably modify the properties. As pointed out in the preceding chapter, the enzyme property is one of the most easily modified of the properties of such materials, so that enzyme action may be destroyed even without experimental evidence of other changes. While much can be done in the study of purified or modified biochemical materials, in order to obtain evidence of the properties of the substances as they exist in living matter, as nearly as possible, their properties and reactions must be followed before such changes have taken place.

A common characteristic of enzyme preparations is their inactivation by heating in aqueous solution. This property is sometimes used as one of the criteria as to whether a given reaction involves an enzyme action. There is no one temperature at which inactivation of all enzymes occurs, but the temperature, the time of heating, the presence of other substances, all influence the rate of inactivation. Unquestionably, such inactivations are due to chemical changes within the molecules, and other changes, such as coagulation, etc., are secondary.

This inactivation by heat of practically all enzymes also necessitates the conclusion that even at comparatively low temperatures gradual loss of activity occurs in solution. It is therefore advisable to keep enzyme solutions at low temperatures, for them to retain their activity as far as possible. No other chemical or physical change has been observed in many of these inactivations.

The presence of the substrate with the enzyme seems to protect the latter from inactivation by heat to a certain extent. As pointed out in Chapter V, this is undoubtedly due to compound formation of enzyme and substrate.

With regard to temperature effects, as a rule enzymes show their greatest accelerating actions at temperatures in the neighborhood of 40°. At higher temperatures it is probable that inactivation of the enzyme takes place with sufficient rapidity to cause apparent decrease in catalytic action.

It has frequently been observed that inorganic salts influence enzyme actions very markedly in some cases. (It may be recalled that recent studies have shown that inorganic salts influence the color changes of indicators, and that different indicators may act differently.) With any one enzyme, addition of a salt may accelerate or retard the action or have no effect. It is impossible to predict what the action will be, but on the other hand, the actions are sometimes so striking that they would appear to offer the most direct clue to the chemical nature of the enzyme. For example, the action of cyanide on the proteolytic enzyme papain is very large.[24] Also, the activating effect of bromide on amylase is great, differing in this respect from chlorides and iodides.[25] The action of manganous sulfate on castor beans, activating the lipase, may also be referred to.[26]

In all studies with the addition of inorganic salts, the hydrogen ion concentrations should be followed carefully. This can be shown from some results on sucrase found by Fales and Nelson.[27] At the optimum hydrogen ion concentration of yeast sucrase action, the addition of sodium chloride had practically no effect on the velocity of the hydrolysis of sucrose. At all other hydrogen ion concentrations, sodium chloride exerted an action, greater progressively as the acidity or alkalinity was increased. The presence of the salt decreased the actions. The authors point out that this raises the important question that the use of buffers in large concentrations for regulating the hydrogen ion concentration of the sucrase solution introduces more or less of an error if it is desired to get the maximum activity of sucrase corresponding to a given concentration of hydrogen ion. This point is of extreme importance in the study of the actions of salts on all enzymes at definite hydrogen ion concentrations, as possible salt actions may result in entirely incorrect conclusions being drawn from work accurate in every respect.

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[24] E. M. Frankel, *J. Biol. Chem.* *31*, 201 (1917).

[25] A. W. Thomas, *Jour. Amer. Chem. Soc.* *39*, 1501 (1917).

[26] E. Hoyer, *Z. physiol. Chem.* *50*, 414 (1907); Y. Tanaka, *Orig. Com. 8th Intern. Congr. Appl. Chem.* *11*, 37 (1912); K. G. Falk and M. L. Hamlin, *Jour. Amer. Chem. Soc.* *35*, 210 (1913).

[27] H. A. Fales and J. M. Nelson, *Jour Amer. Chem. Soc.* *37*, 2769 (1915).

The hydrogen ion concentration has not been determined in many such salt studies, and although this introduces an element of doubt in the conclusions, still some interesting deductions are possible. For example, the results of the study of the actions of a number of neutral salts on the action of a castor bean lipase preparation toward ethyl butyrate[28] may be quoted. In every case, the change in activity, whether increase or decrease, was found to be a continuous function of the concentration of the salt added. Decreased activities, as compared with the aqueous solutions, were shown by all the uni-univalent salts, by the chlorides and nitrates of barium and calcium (except for the most dilute solutions) and magnesium, by sodium oxalate, and by dilute solutions of sodium sulfate. Increased activities were shown by dilute solutions of the chlorides of barium and calcium, by more concentrated solutions of sodium sulfate, by magnesium sulfate, and by the chloride and sulfate of manganese. Potassium sulfate caused no change. It is possible, by a careful study of these results, to point out series of regularities. For instance, for the sodium and potassium halides, the retardations increased in the order chloride, bromide, iodide, fluoride. Terroine[29] had found the same order for the actions of the sodium halides on pancreatic lipase. The lithium salts exerted greater retarding actions than did the sodium and potassium salts. Such regularities might be multiplied, the positive and negative constituents of each salt, or possibly the ions, apparently exerting their individual actions in each case, which sum up to give the total action. A number of investigations of the actions of salts on enzymes might be quoted, all carried out in an analogous manner. A certain number of regularities might be deduced from each investigation, but unfortunately, all suffer from the lack of exact control of the hydrogen ion concentrations of the mixtures. The results can therefore be considered of value only from a qualitative point of view, and while unquestionably indicating certain facts of interest, at present do not appear to lend themselves to the development of the more exact chemical studies which are needed for a systematic following up of the enzyme problem.

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[28] **K. G. Falk,** *Jour. Amer. Chem. Soc.* **35,** 601 (1913).

[29] **E. F. Terroine,** *Biochem. Z.* **23,** 429 (1910).

It has been stated that serum albumin or charcoal or glass beads inhibited the actions of certain enzymes such as sucrase.[30] Griffin and Nelson[31] showed that these inhibiting actions did not occur with sucrase if the hydrogen ion concentrations were kept unchanged, and that the action of these added substances caused the retardations in the same measure that they changed the hydrogen ion concentrations.

The interpretation of the action of salts is not easy. The treatments are in the main very simple. There seems to be little chance for any deep seated chemical change to take place to bring about such marked activations or inactivations as are observed at times. That some change takes place is unquestionable. Again the similarity in the marked color changes of indicators brought about by simple treatments and the marked changes in chemical actions of enzyme preparations brought about by simple treatments brings to mind the possibility of a similar underlying chemical transformation or change being responsible for both. This change would involve some intramolecular rearrangement. This question will be taken up in more detail in the next chapter.

An important feature of enzyme actions is the specificity, each enzyme accelerating a more or less definite reaction. Thus, the soy bean urease accelerates the hydrolysis of urea to a very marked extent, but has very little influence on the hydrolysis of methylurea. Sucrase hydrolyzes sucrose and not maltose; maltase the reverse. These examples might be multiplied indefinitely, but for such detailed information the reader is referred to the larger textbooks dealing with the detailed reactions.

E. Fischer's lock-and-key simile[32] for the mutual getting together of substrate and enzyme, each fitting in with the other, gives a mechanical picture of the action. The actions would evidently depend then upon a combination of enzyme and substrate followed by a breaking down in a different way to regenerate the enzyme and the products of the hydrolysis.

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[30] E. Beard and W. Cramer, *Proc. Roy. Soc. London (B)* 88, 575 (1915); A. Erikson, *Z. physiol. Chem.* 72, 313 (1911).

[31] E. G. Griffin and J. M. Nelson, *Jour. Amer. Chem. Soc.* 38, 722 (1915).

The specificities of enzyme actions have always aroused the greatest interest. The relations have been considered frequently to be peculiarly characteristic of enzyme actions, but the time appears to be ripe to take a somewhat more moderate view of the question. The specific actions of enzymes vary to a certain extent. Some enzymes, such as urease, are apparently limited in their actions to one substance, others exert their influences on groups of substances. For example, α-glucosides are hydrolyzed by maltase, β-glucosides by emulsin, many proteins by pepsin, many peptides by erepsin, many esters by lipase, etc. There are variations with regard to the extent of the actions within each group as well. On the other hand, the specific actions of enzymes, while interesting in every case and striking in many, are not unique among chemical reactions. Systematic qualitative analysis shows many just as interesting and (to the writer) just as striking reactions involving specificity as the reactions of enzyme chemistry. This question will be taken up again in Chapter X.

VII.—Chemical Nature of Certain Enzymes

After treating of the physical and chemical properties which are to a certain extent common to enzyme preparations, the next question in the natural sequence of development would involve the actual chemical nature of certain specific enzymes. It may be stated at once, in order to forestall any misunderstanding, that there is no proof that any enzyme has been obtained in a state of purity as a chemical individual is so considered. The question may even be raised as to whether a chemical molecule possessing a definite molecular weight and arrangement of constituent atoms is present in any enzyme preparation, conferring upon the preparation the properties which are included under enzyme actions. The significance of this statement will be developed further in this chapter.

The study of the chemical nature of enzymes is complicated in most cases by reason of the complexity of the substances whose changes they accelerate. This difficulty can be obviated for a few of the enzymes. For example, the lipases and esterases accelerate the hydrolysis of fats and esters. While the mechanism of the reaction of hydrolysis of an ester to form alcohol and acid in the absence of lipase has not been solved in all respects, much light has been thrown on the reaction and the theoretical views placed upon a comparatively firm foundation. The compositions and properties of the initial and final products undergoing the enzymatic change are known. This eliminates one of the unknown factors which complicate the study of so many of the enzyme reactions.

As stated before, practically all enzymes are colloids or are intimately associated with substances having colloidal properties. Furthermore, as described in Chapter VI, in most cases it seems that the enzyme is associated with protein matter, either as an essential part of the protein molecule or accompanying it in such a way that separation has not yet been effected without completely destroying the enzyme action.

These facts make it evident that for the case of lipase, to use a specific example, the isolation of the enzyme in a pure state is a phase or part of the problem of the isolation of a pure protein, since in the separation of the active lipase from inactive material present with it, the resulting bodies approach in properties and composition those which are generally taken to typify proteins. In the problem of isolating pure proteins, it has been possible by careful treatment to obtain bodies having the same properties at different times. This is somewhat different from obtaining a pure protein possessing the same properties as when present in living matter. The operations involved in such isolations are always sufficient to change the properties of the protein to some extent. The problem of isolating a pure lipase, for example, must wait therefore for the solution of the problem of the isolation of proteins possessing the properties which they exhibit in living matter, using the term living to include also matter showing the actions of enzymes.

If, therefore, the solution of the problem of isolating a pure enzyme does not show a hopeful outlook at present, considering also the colloidal nature of the material with which it is necessary to work, there is a possibility of attacking the problem in a somewhat different way. An enzyme, as a rule, accelerates a more or less specific reaction or group of reactions. Considering the very complex nature of the protein or other molecule which includes the enzyme, or with which the enzyme may be associated, and the more or less specific reaction which it accelerates, it would appear that a reasonable assumption would consider that some definite grouping in the complex enzyme molecule is responsible for a given enzyme action. The problem would therefore resolve itself from this point of view into a study of the chemical nature of such a grouping.

The enzyme, lipase, was studied in this way, and the results found are of interest as showing a possible solution of the chemical nature of this enzyme. Some space will therefore be devoted to this phase of the problem and the attempt at its solution.[1]

This lipase work was carried out in the main with preparations from castor beans, although other sources were also used. There has been a general tendency in the study of enzyme actions to attempt to attain conditions under which the enzyme would show a maximum action. This method of studying the problem is likely to introduce a number of new complicating factors, so that it was considered that if the action was due to some definite grouping, a study of the factors which caused a loss of the action might aid in throwing light on its nature. A systematic study of the factors which caused inactivation of the esterase and lipase was therefore undertaken. The results were presented in detail elsewhere.[2]

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[1] K. G. Falk, "A Chemical Study of Enzyme Actions," *Science* 47, 423 (1918).

Inactivation of lipase and esterase preparations was brought about by acids, bases, neutral salts, alcohols, acetones, esters and heat.

The different ways in which these preparations may be inactivated make it appear at first sight as if different reactions occur in the inactivations. If, however, a definite chemical group is responsible for a definite enzyme action, it might perhaps be more reasonable to assume that inactivation follows a definite reaction. The preparations used were essentially protein in character. There was no evidence that a dehydration, or loss of the elements of water, caused inactivation. Some of the reactions indicated that a possible hydrolysis might be a cause of inactivation. With proteins, hydrolysis is generally taken to occur with the —CO—NH— group, the peptide linking, which goes over into the carboxyl and amino groups. Experiments with all the inactivations in no case showed an increase in the formol titration such as would be expected in this reaction, and, therefore, makes the assumption of such a hydrolysis improbable. Coagulation of the material accompanied some of the inactivations. This physical change alone does not appear satisfactory as an explanation; some change in chemical structure unquestionably must accompany or produce the physical phenomenon. Also, the lipase material in water suspension showed the same activity as in 1.5 normal sodium chloride solution.

The explanations of the chemical changes accompanying inactivation so far suggested are not satisfactory. The reagents used are simple. It is difficult to conceive of a very deep-seated chemical reaction taking place under so many different conditions, none of a complex nature. The only chemical change which appears probable under these conditions is that involving a simple rearrangement within the molecule, such as a tautomeric (or perhaps better, desmotropic) change involving in the simplest case the change in position of a hydrogen atom. In considering the structure of proteins it is evident that such a rearrangement is possible in the peptide linking.

The hypothesis suggested is that the active grouping of the esterase and lipase preparations is of the enol-lactim structure, —C(OH)=N—, the specificities being dependent in part upon the groups combined with the carbon and nitrogen, and that inactivation consists primarily in a rearrangement to the keto-lactam group, —CO—NH—.

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[2] *J. Biol. Chem. 31,* 97 (1917).

This hypothesis was tested in several different ways. It has been found that in tautomeric substances, the presence of alkali in solution favors the enol form of compounds showing such tautomerism, while acid favors the existence of the keto form. The hydrolytic actions of some simple dipeptides on esters at different hydrogen ion concentrations would, therefore, be evidence bearing on this point, the alkaline solutions presumably favoring the enol-lactim structure. In order to find the actions exerted by the amino-carboxyl groups of the peptide, the hydrolytic actions of a number of aminoacids on different esters were determined under similar conditions at the same hydrogen ion concentrations. The actions of the dipeptides and aminoacids were also measured with the actions of the amino-carboxyl groups masked by the hydrogen of the carboxyl group being replaced by the ethyl group, and also by testing compounds such as hippuric acid, which do not contain an amino group.

In these compounds, it is possible that the equilibrium between the keto-lactam enol-lactim forms might be changed rapidly if the conditions were changed slightly. A more stable substance was therefore studied from this point of view. Imido esters, as shown by the formula (a), possess the enol-lactim structure in which the hydrogen atoms may be substituted by organic radicals. The hydrolytic actions on esters of ethyl imidobenzoate (b) at different hydrogen ion concentrations and under various conditions were measured.

$$\underset{(a)}{R{-}C(OR') = NR''}, \qquad \underset{(b)}{C_6H_5{-}C(OC_2H_5) = NH}$$

Finally, in order to reproduce the conditions and properties of naturally occurring lipases as far as possible, a number of different proteins were treated with alkali for the purpose of producing an enol-lactim grouping in the peptide linking if this were possible, then neutralized to different hydrogen ion concentrations and the hydrolytic actions tested on a number of different esters.

The following table shows some of the experimental results obtained. The determinations were made by titration with alkali, all necessary corrections for blanks being introduced. The actions were then calculated in terms of equivalents of acid × 10^{-4} (as titrated with 0.1 N alkali solution) formed from 1 gram equivalent of the ester by the action of 0.1 gram substance in 24 hours at 38°. The relative results were then calculated with the ester showing the greatest amount of hydrolysis put at a value of 100.

Selective Actions of Ester-Hydrolyzing Substances

	Glycine[3] pH 6.1	Castor Beans[4]	Glutamic Acid[3]	Aspartic Acid[3]	Glycyl glycine[4] 9.0	Imido Benzoate[4] 7.0	Casein[5] 8.0	Gelatine[5] 8.0	Egg Albumin[5] 9.0
Phenyl acetate.....	100	100	30	16	100	100	0	46	100
Methyl acetate.....	44	38	100	100	60	90	37	36	46
Glyceryl triacetate.	71	82	50	59	24	36	49	28	85
Ethyl acetate......	83	22	72	79	46	83	36	39	0
Ethyl butyrate.....	20	48	17	11	15	33	0	4	0
Methyl benzoate...	...	...	...	...	5	14	100	0	0
Ethyl benzoate....	19	6	12	6	0	0	0	100	68
Phenyl benzoate...	5	3	4	4	0	0	...	...	...
Olive oil..........	...	0	...	...	...	...	2	11	0
Cottonseed oil.....	...	...	...	...	...	...	0	...	0
Castor oil.........	...	6	...	...	...	...	...	...	...

[3] M. L. Hamlin, *Jour. Amer. Chem. Soc.* 35, 1897 (1913).

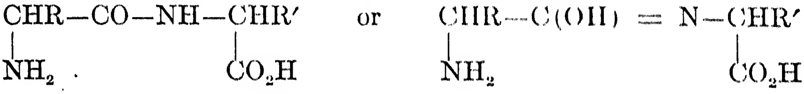
[4] K. G. Falk, *J. Biol. Chem.* 31, 97 (1917).

[5] F. Hulton-Frankel, *J. Biol. Chem.* 32, 395 (1917).

These results show one of the most striking characteristics of the actions, that is, their selectivity. Different substances showed markedly different actions on the esters. It is not surprising in the first instance to obtain hydrolytic actions, but the variations are interesting. The chemical configurations may also be considered somewhat further. In doing this, the further experimental results found will not be given in detail but will only be referred to.[6]

With regard to hydrolytic actions of a number of the simpler peptides on esters, the actions were very much more marked in the alkaline solutions than in the neutral. At pH 9.0 for example, considerable action was obtained, especially toward the acetates. There was in these cases a decrease in the hydroxyl ion concentration in the course of the experiments, approaching neutrality or going beyond in some cases. It is difficult to judge how far this influenced the results, as it undoubtedly did. The objection may be raised that the alkalinity of the solutions alone caused the hydrolysis, and that the peptides acted only as buffer mixtures to keep the hydroxyl ion concentration predominant as compared to the water-ester blanks, which in some cases became neutral or slightly acid in reaction more rapidly. This objection is met by comparison with the results obtained with the aminoacid solutions where the buffer action was essentially the same, but entirely different actions were obtained, both absolutely and relatively. A comparative study of the hydrolytic actions of the dipeptides in themselves also met the objection.

The general formula for the peptides may be written as follows:



$$\underset{\mathrm{NH_2}}{\mathrm{CHR}}-\mathrm{CO}-\mathrm{NH}-\underset{\mathrm{CO_2H}}{\mathrm{CHR'}} \quad \text{or} \quad \underset{\mathrm{NH_2}}{\mathrm{CHR}}-\mathrm{C(OH)}=\mathrm{N}-\underset{\mathrm{CO_2H}}{\mathrm{CHR'}}$$

The groups which may be considered to be involved in the hydrolytic actions are the amino and carboxyl groups or the central —CO—NH— group or its tautomer. In attempting to separate the actions of these groups, two lines of experimentation were followed. In the first place, the actions of the amino and carboxyl groups were masked by using glycylglycine ester hydrochloride and hydrobromide; and secondly, the actions of the amino and carboxyl groups alone were followed by studying aminoacids, all under the same conditions under which the peptides were studied. While these methods permit the studying of the groups alone, they leave out of account the possible factor of the influence on the tautomeric equilibrium of the molecule as a whole.

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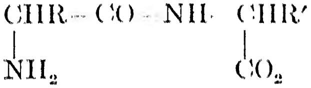
[6] For experimental details and data cf. K. G. Falk, *J. Biol. Chem.* *31,* 97 (1917).

The results showed very marked actions at pH 8 and 9, but the solutions became neutral rapidly (presumably in this way reverting back to the keto-lactam structure). A significant feature was the lack of action toward ethyl butyrate, and the comparatively large action toward glyceryl triacetate.

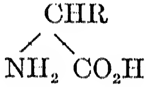
The second method of studying the influence of the different groups separately was to compare the actions of some aminoacids with the peptides under similar conditions. This may be illustrated by comparing the following formulas for aminoacids and peptides.

(A)



$$\underset{NH_2}{\underset{|}{CHR}}-CO-NH-\underset{CO_2}{\underset{|}{CHR'}}$$

(B)



$$\underset{NH_2 \quad CO_2H}{\underset{\diagup \ \diagdown}{CHR}}$$

By comparing the actions of equivalent amounts of substances of Formulas A and B under comparable conditions, it should be possible to find the action due to the grouping —CO—NH— in A with the possible reservation that this group and the amino and carboxyl groups may exert reciprocal influences upon each other although no direct evidence of such influence has been obtained. The results obtained in an extended series of experiments led to the following conclusions: Under the conditions used the ratios of the actions toward glyceryl triacetate and ethyl butyrate of the simple aminoacids was close to unity, while for the dipeptides it varied from 5 to 12 to one. This proves that the actions were not due to the hydroxyl ion concentrations, but that the aminoacids and peptides are the important factors. The hydrolytic action of one gram molecule of glycine toward the different esters was calculated from the experimental results and may be given in terms of the amount of acid in tenths of millimols formed at 38° from 1.0 cc. of ethyl butyrate, 0.5 cc. of glyceryl triacetate, etc. Toward ethyl butyrate for 26 hours' action it was found to be 6.1×10^2; for 45 to 46 hours 8.2×10^2 (mean); toward glyceryl triacetate it was found to be 8.4×10^2 for 26 hours' action, and 10.8×10^2 for 45 to 46 hours. With dipeptides, the mean actions found for 19 hours were 6.2×10^2 toward ethyl butyrate and 46.4×10^2 toward glyceryl triacetate. This indicates that the action of the dipeptides toward the ethyl butyrate was due mainly to the amino and carboxyl groups and confirmed the results obtained with the glycylglycine ester hydrogen halides. Subtraction of the aminoacid glyceryl triacetate value from the value of the peptide left an action of 35.8×10^2 to be accounted for by the group —CO—NH— or

—C(OH) = N—. The mean value found with the peptide ester hydrogen halides was 17.2×10^2 but the difference may well have been due to the more rapid neutralization and accompanying shift in the tautomeric equilibrium with the latter.

The actions of the simpler aminoacids toward different esters were treated in detail by Hamlin[7] and it was shown by him that if the esters were arranged in a series according to the extent of their hydrolyses, different arrangements resulted with the different aminoacids for the same hydrogen ion concentration and different as well from an isohydric solution containing no aminoacid.

Since the keto-lactam group is present in other substances besides peptides, experiments were carried out to find whether these exerted any hydrolytic actions on esters. Urea gave no action whatsoever at hydrogen ion concentrations between 10^{-4} and 10^{-10} N. Hippuric acid gave very small actions at $10^{-9.0}$ N. It is therefore evident that the structure of the compound as a whole is of importance in determining the equilibrium between the tautomeric forms, if these be involved in the actions.

In the table, the results with an imido ester were given. Imido esters contain the enol-lactim structure, possessing the general formula R—C(OR′) = NR″. The one studied in this work was ethyl imidobenzoate, $C_6H_5 - C(OC_2H_5) = NH$. The action toward ethyl butyrate was found to be small. The actions toward glyceryl triacetate were comparatively large, however. A maximum action was observable at pH 8, compared with more acid or more alkaline solutions. The results toward different esters calculated as for the dipeptides, at pH 7.0 gave the same order of decreasing actions as for glycylglycine at pH 9.0, except that the positions of the first two members, glyceryl triacetate and phenyl acetate, were interchanged. This shows a marked similarity in behavior, while the minor difference may be due to secondary differences in structure. The results toward glyceryl triacetate with the imido ester, especially at pH 7.0 to 10.0, were not far removed from the results for the dipeptides at pH 9.0. Since the action toward ethyl butyrate was small or negligible, it is evident that the actions observed confirm the view that the action is due to the grouping —C(OR) = N—.

An interesting parallelism between the imido ester and the naturally occurring lipases is the fact that the former is inactivated by acids, by bases, by heating in solution, and by long standing in solution.

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[7] M. L. Hamlin, *Jour. Amer. Chem. Soc.* **35**, 1897 (1913).

This inactivation refers to the ester-hydrolyzing action and the change is accompanied chemically by the hydrolysis of the (OR) grouping followed by the tautomeric rearrangement of the amidine complex to the acid amide, and probably hydrolysis of the latter. The optimum hydrogen ion concentration for the imido ester is also interesting. This may be due to its more rapid decomposition in more acid or more alkaline solutions.

In order to determine whether or not the conditions which are known to favor the enol-lactim grouping in simple substances will produce ester-hydrolyzing groups or substances from proteins, an investigation was carried out in which a number of proteins (fifteen in all) were treated with alkali of different strengths and under different conditions, and after neutralization to various points tested for ester-hydrolyzing actions.[8] Some of the results are shown in the table and the general conclusions arrived at were as follows: The time and temperature at which the alkali stood in contact with the protein did not seem to make much difference in the activity of the solution except where the temperature was quite high (80° C.). With regard to the concentration of alkali, for casein, gelatine, and egg albumin, 3 N alkali seemed to produce solutions of highest activity. The solutions showed tendencies toward optimum acidity conditions, though not very marked. Activity was greater in slightly alkaline solution. When hydrolysis of the protein was accomplished by acid instead of by alkali, the solutions, when treated similarly, did not possess ester-hydrolyzing properties.

In this discussion of the active grouping in lipase actions, the experimental work was limited almost entirely to the peptide linking occurring in proteins. It is evident, however, that such tautomeric or desmotropic structures, enol-lactim and keto-lactam, may be present possibly with the hydrogen of the hydroxyl substituted by a hydrocarbon or other radical in other groupings, and the results of this investigation in no way limit the activity to the peptide linking. In view of the complexity of the protein molecule, it is highly probable that such structures are present and rearrangements possible with other groups and that the specificities are in part dependent upon these.

It may be pointed out in this connection that in a paper entitled "The Catalytic Action of Amino-Acids, Peptones, and Proteins in

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[8] **F. Hulton-Frankel, *J. Biol. Chem.* 32, 395 (1917).**

Effecting Certain Syntheses,"[9] Dakin showed that a number of condensation reactions of organic substances, some of them possibly analogous to some occurring in the living cell, are accelerated by amino-acids, peptones, albumoses, and even proteins as catalysts. This work is most suggestive and makes it appear probable that many more such reactions will be found, especially now that conditions can be controlled more satisfactorily than when Dakin carried out his experiments.

In how far the conclusions reached with lipase may be applied to other enzymes is a question. It seems probable, because of the comparatively simple treatments by which most enzymes may be inactivated and also activated, that with them also a simple rearrangement or perhaps desmotropic change is connected with loss or gain in activity. There is, however, no reason to suppose that the active grouping is the same for all enzymes. Each enzyme must be studied separately and the conclusions as to the chemical nature of one active enzyme grouping can not, without further evidence, be applied to an enzyme grouping connected with a different action.

The fact that the hypothesis with regard to the active grouping in lipolytic action gave positive results must be looked upon as a fortunate guess. Though there is no direct proof that the active lipase grouping is the one indicated, a number of indirect lines of evidence point to the same conclusion and this group may be accepted as the enzymatically active one, until there is a more satisfactory explanation, or direct evidence to the contrary. The analogy of the imido ester and the naturally occurring lipases is striking. Unquestionably a study of the hydrolytic actions of a number of different imido esters and their substitution products upon different esters, will give results showing as great differences and specificities as the naturally occurring lipases.

Further, it seems fairly certain from the evidence presented here and in Chapter VI, that inactivation of lipase preparations, and in all probability of all other enzyme preparations, depends upon simple tautomeric changes or rearrangements, reversible in some cases, irreversible in others, within the molecule, and that the specificity depends in part upon the various atoms or groups, their natures and arrangements, combined with the active grouping and to a minor extent upon those atoms or groups present in the rest of the molecule, and in part possibly upon the action of the substrate upon the enzyme preparation as will be developed in the following chapter.

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[9] H. D. Dakin, *J. Biol. Chem.* 7, 49 (1909-1910).

The view that an enzyme preparation is made up of or contains an active grouping or substance, unstable to a certain extent, which is stabilized by another grouping or substance, possibly colloidal in nature, was suggested earlier by Perrin[10] and also by Röhmann and Shmanine[11] in connection with peroxidases. Mathews and Glenn[12] considered enzymes to be a combination of a colloid with an active principle. The former was thought to be related in nature to the substrate, while the latter in the case of sucrase and diastase was considered to be protein.

The properties of oxidizing enzymes have often been referred to the content of iron or manganese of these preparations. Similar changes have been brought about by horseradish peroxidase and by a trace of ferrous sulfate, either of which accelerates greatly the oxidation reaction between lactic acid and hydrogen peroxide. This thought was followed up by Dony-Hénault[13] who prepared an artificial oxidase by repeated solution in water and precipitation with alcohol of a mixture of 10 parts gum arabic, one part manganous formate and 0.4 part crystallized sodium bicarbonate. The colloidal complex containing manganous hydroxide showed the property of accelerating oxidation reactions. The gum arabic evidently stabilized the manganous hydroxide so that it could continue its action. It is a question, however, in how far the remaining properties of this preparation parallelled those of the naturally occurring enzymes.

The chemical study of an enzyme, peroxidase, was taken up in a different way by R. Willstätter and A. Stoll.[14] These chemists studied the following questions: (1) whether enzyme activity is possessed by an analytically pure compound or whether an "enzyme" is a system of co-operating substances; (2) whether a metal is an integral part of the enzyme; and (3) what atomic groupings are associated with enzyme activity. The horseradish peroxidase was studied. A quantitative method for determining the activity was developed based upon the oxidation of pyrogallol to purpurogallin in the presence of hydrogen peroxide.[15] The method of separating the active enzyme material may be given in detail since it is representative of the methods in use in such work. Thin slices of horseradish roots (5 kg.) were kept for a few days in flowing water in order to remove the simpler products by dialysis through the cell walls. The washed material was digested with oxalic acid solution (40 g. to 10 l.) for a few hours. By this means, the regulating influence of the living protoplasm was removed, the peroxidase was precipitated, apparently adsorbed on coagulated protein material, and dialysis proceeded further, mustard oil being extracted in large quantities. So extensive was the dialysis, that the dried slices lost more than 25% in weight and half of their mineral matter. The material was crushed in a mill, washed on a filter with about 5 liters of water containing 1.5 g. of oxalic acid, and then thoroughly pressed free from sap. The residue (1.5 kg.) was intimately triturated with barium hydroxide almost sufficient to overcome its acidity, then pressed again, and then treated with further quantities of barium hydroxide to liberate the enzyme. Most of the barium was retained by the fibers, the expressed juice just acidified with carbon dioxide to remove the remainder, and the filtrate then mixed with 0.9 of its volume of alcohol. Slimy substances were precipitated and the filtrate evaporated to 50-70 cc. in vacuo from a bath at 50°. The residue was filtered again, and then mixed with 5 volumes of alcohol, whereby the crude enzyme was precipitated. This was purified a little more by redissolving it in water containing a trace of sulfuric acid and reprecipitating it by alcohol. The crude material was found to be a mixture of the enzyme and a nitrogenous glucoside, which could be separated by precipitation as a compound with mercuric chloride. Accordingly, an aqueous solution was treated with 0.5% mercuric chloride and a trace of calcium chloride to coagulate the jelly-like double compound, the mass filtered, and the enzyme reprecipitated by alcohol from the filtrate. The peroxidase material was then dissolved in water, whereby some of the mercuric chloride compound remained undissolved, the clear solution obtained by centrifuging, reprecipitated by alcohol and the process repeated until the enzyme formed a clear solution in water. From 5 kg. horseradishes, the best preparation showed a yield of 0.45 gram containing 60% of the original enzyme and an activity 2,700 times that of the original material. The final enzyme preparation was mainly a nitrogenous glucoside, containing a pentose (more than 30%) and an equimolecular quantity of another sugar, probably a hexose. 5.5% of mineral ash, mainly alkaline earths and iron, was present. The iron did not appear phorus was combined with the polysaccharide, and the two could be separated from the nitrogenous constituent. Mannose and glucose were obtained from the polysaccharide. The nitrogen was present mainly as protein. The usual distribution of the nitrogenous constituents of a protein were found with perhaps a greater percentage of soluble aminoacids present than in the average run of proteins.

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[10] J. Perrin, *J. Chim. Phys.* 3, 50 (1905).

[11] F. Röhmann and T. Shmanine, *Biochem. Z.* 42, 235 (1912).

[12] A. P. Mathews and T. H. Glenn, *J. Biol. Chem.* 9, 29 (1911).

[13] O. Dony-Hénault, *Bull. de la Classe de Sciences, Acad. Roy. de Belgique,*

The study of the sucrase preparation unfortunately has given no information with regard to the chemical nature of the active enzyme grouping or molecule. In view of the apparently purified condition of the preparation, its great activity, and ease of handling, this is a remarkable circumstance, and points to the elusive character of the chemical configuration of the active enzyme.

An extended investigation on the amylases from typical animal, vegetable, and fungus sources has been carried out by H. C. Sherman and his co-workers.[17] The methods used by earlier workers were adopted and improved upon. The outlines of the methods used for obtaining the final enzyme materials are as follows: For pancreatic (animal) amylase,[18] pancreatic powder was extracted with 50% alcohol, filtered, poured into 7 volumes of an alcohol (1 part) ether (4 parts) mixture, decanted, dissolved in water and precipitated with 5 volumes of absolute alcohol. The precipitate was dissolved in 50% alcohol (containing maltose to stabilize the enzyme) dialyzed in collodian bags against 50% alcohol, filtered and precipitated with alcohol and ether. For malt (vegetable) amylase,[19] the material was dissolved in water, dialyzed, precipitated with saturated ammonium sulfate, filtered, dissolved, dialyzed, inactive material precipitated by addition of alcohol to make a 50% solution by volume, then active material precipitated with alcohol to make a 65% solution. For takadiastase (fungus, *Aspergillus Oryzae*) amylase,[20] the method used consisted essentially in extracting with water, precipitating with ammonium sulfate, dialyzing, and finally precipitating fractionally with alcohol.

It was found necessary to keep the temperature fairly low in these preparations because of the otherwise rapid deterioration of the enzyme.

The methods of determining the actions of the amylases on soluble starch were studied carefully. The amyloclastic (starch splitting, disappearance of blue color with iodine) and saccharogenic (formation of substances reducing cupric salts) actions were compared.[21] Differences found by the two methods of testing were doubtless due to the fact that "saccharogenic power" refers to the amount of maltose produced and "amyloclastic power" refers to the amount of starch all of which is digested to a certain point within a certain time. The latter method may therefore give misleadingly low results. A number of starches from different sources were also studied but showed no differences in behavior.[22]

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[17] Series of papers published in *Jour. Amer. Chem. Soc.* during the past ten years.
[18] H. C. Sherman and M. D. Schlesinger, *Jour. Amer. Chem. Soc. 34,* 1104 (1912).
[19] H. C. Sherman and M. D. Schlesinger, *Jour. Amer. Chem. Soc. 37,* 643 (1915).
[20] H. C. Sherman and A. P. Tanberg, *Jour. Amer. Chem. Soc. 38,* 1638 (1916).

The properties of the pancreatic and malt preparations may be compared. With regard to the two methods of testing, the malt enzyme showed that the iodine reaction persisted even after much maltose had been formed. For instance, after one-half of the original weight of the starch had been transformed into maltose, addition of iodine gave a deep blue color, after two-thirds, the iodine test was violet blue to violet red. On the other hand, with the pancreatic enzyme the iodine test was red when two-fifths to one-half of the original weight of the starch had been transformed into maltose, and no color shown after one-half to three-fifths.

A comparison of the chemical properties of the purified pancreatic and malt amylases showed the following similarities and differences.[23] Both are amorphous substances, containing 15-16% nitrogen, the nitrogen being present in the forms distinguishable by the Van Slyke procedure in proportions within the range of variation shown by other protein substances. Both are soluble in water and in 50% alcohol, insoluble in concentrated alcohol or in acetone. Both, when heated in aqueous solution yield a coagulated albumin and a protease or peptone. Both deteriorate during dialysis, much more rapidly at room temperature than at 5°–10°. The optimum saccharogenic (diastatic) action of malt enzyme was found to be at pH 4.4 ± 0.2; of pancreatic, at 8.0 to 8.5. On soluble starch, the pancreatic enzyme showed much greater activity (2½ times) than did any malt preparation. The purified pancreatic preparation, in contradistinction to the malt preparation, showed pronounced proteolytic action on casein and gelatine. The two products also showed different stabilities on standing in aqueous solution, in 50% alcohol, and on dialysis.

The *Aspergillus oryzae* amylase was similar to the other amylases in general. It showed a lower nitrogen content (10.8%). It possessed greater amyloclastic but smaller saccharogenic actions than did the most active malt preparations.

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[21] H. C. Sherman and M. D. Schlesinger, *Jour. Amer. Chem. Soc.* *35*, 1784 (1913).

[22] H. C. Sherman, F. Walker, and M. L. Caldwell, *Jour. Amer. Chem. Soc.* *41*, 1123 (1919).

[23] H. C. Sherman and M. D. Schlesinger, *Jour. Amer. Chem. Soc.* *37*, 1305 (1915).

It may be stated that the actions of salts on the activities of these amylase preparations were also studied carefully. For example, the removal of salts did not inactivate the malt enzyme completely as it did the pancreatic enzyme.[24] Further, with malt, the amyloclastic action reaches an optimum at concentrations of the activating agent (salt) much below that which gives the optimum saccharogenic action. In other words, the former action requires a smaller salt concentration for a maximum than does the latter action.

The separation of the esterase and lipase materials in castor beans may be referred to briefly.[25] The former is soluble in water, insoluble in moderately concentrated salt solution, the latter insoluble in water, soluble in salt solution. To obtain the desired enzymes, husk and oil free castor beans may be extracted with water, dialyzed and filtered. A clear solution is obtained in this way. The longer the time of dialysis and the higher the temperature of the water, the greater the loss in activity. Precipitated with acetone, the solid preparation showed a certain activity in some cases when dissolved again. It seemed to be essentially protein. The residue from the water extraction was extracted with 1.5 N sodium chloride solution. More concentrated salt solution gave a larger amount of extracted material but smaller enzyme action. The salt extract was dialyzed. Length of time of dialysis had no effect on the activity. The precipitated material contained the active enzyme, but if this was filtered, washed with acetone or alcohol, all activity was lost. Sodium fluoride retarded the actions, other salts, including manganous sulfate, had no effect.

The actions of these two preparations under comparable conditions on ethyl butyrate and glyceryl triacetate gave results which indicated that the water soluble enzyme may be designated as an esterase, the salt soluble as lipase. Both preparations acted on both esters, the difference being one of relative amount of action.

The chemical analyses of the two solid preparations already spoken of in Chapter VI showed them to be protein in character.

It may also be mentioned that the water insolubility of the castor bean lipase does not mean that all lipases show this property. For instance, the soy bean lipase was found to be soluble in water.[26] On the basis of the views advanced earlier in this chapter, this means only that the active enzyme grouping is combined in different complexes which show varying properties in the two cases.

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[24] H. C. Sherman and A. W. Thomas, *Jour. Amer. Chem. Soc.* 37, 623 (1915).
[25] K. G. Falk and K. Sugiura, *Jour. Amer. Chem. Soc.* 37, 217 (1915).
[26] K. G. Falk, *Jour. Amer. Chem. Soc.* 37, 649 (1915).

A study of the chemical changes in the purification of pepsin was published recently.[27] The general method of purification involved fractional precipitation, followed by salting out, filtering, and dialyzing. The results indicated that the purification consisted in the elimination of secondary protein derivatives, including aminoacids.[28] Calcium and sulfur appeared to be unaltered as a result of purification, but phosphorus was reduced in amount. Chlorides apparently were entirely removed. No material change in optical activity was observed. The pepsin tended to approach nearer to the actual character of a protein (possibly a glycoprotein) with increasing proteolytic activity. Ringer [29] also obtained pepsin which contained no chlorine or phosphorus after purification and which retained its activity.

The description of the various enzyme preparations in the latter part of this chapter does not add to the knowledge of the chemical nature of the active enzymes. However, a knowledge of the methods by which such preparations may be obtained is essential, and it is for this reason that they have been included in this form. The methods used in the different cases follow closely those used in obtaining substances of biochemical origin in general. It is possible with enzyme preparations to keep a close check on the changes which may take place in the various manipulations by means of the enzyme actions which may reflect changes not detected by more usual chemical methods. The choice of reagents used is also limited to a certain extent by their possible action on the enzyme. However, the examples given should be sufficient to illustrate the methods which have been used in the past and which may be used with new materials, bearing in mind the limitations which have been indicated.

A practical point in such methods must not be overlooked. The possibility of bacterial growth in the preparations must be excluded rigidly, both in obtaining enzyme material and in testing its activity, as otherwise it would be impossible to determine whether a given chemical reaction is due to bacteria or to the enzyme under investigation. Since it is extremely difficult to keep the material which is generally used in a sterile condition, it is necessary to employ substances which will prevent bacterial growth. A great number have been suggested and used including toluene, chloroform, tricresol, various salts such as sodium fluoride, cyanides, etc. In every case the possibility exists that the substance added as bactericide or antiseptic may exert a deleterious action on the enzyme, possibly inactivating it. The special substance to be used in any one enzyme investigation must be carefully scrutinized, therefore, at the same time that the use of some such agent must be looked upon as imperative.[30]

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[27] L. Davis and H. M. Merker, *Jour. Amer. Chem. Soc.* *41*, 221 (1919).

[28] This fact was also pointed out by T. B. Aldrich, *J. Biol. Chem.* *23*, 339 (1915).

[29] W. E. Ringer, *Z. physiol. Chem.* *95*, 195 (1915).

[30] Cf. S. Morgulis and V. E. Levine, *Science* 52, 202 (1920).

VIII.—Mechanism of Enzyme Actions

Enzyme actions manifest themselves in the changes in velocity of definite chemical reactions brought about by the presence of certain preparations of biochemical origin. They may be considered to be a group of catalytic reactions. The development of the subject of chemical kinetics has shown how the velocity of a chemical reaction may be measured and used in connection with mathematical expressions, and how general mathematical equations may be deduced which show the law covering the course of the velocity of the change. In Chapter II, some of the simple kinetic equations were given with illustrations of their uses, and an attempt was made to show some of the fundamental concepts upon which the deductions and applications of these equations depend, as well as the possible complications which may arise in their use.

The mechanism of enzyme actions may include such relations as the various steps if the reaction takes place in stages, the factors influencing the velocity of the reaction, the type of products formed, etc. The actual chemical composition and chemical properties of any of the substances taking part are not involved primarily in the discussion. They are, of course, directly responsible for any and all of the phenomena observed in reactions. The main factors to be considered here, however, are the velocities of the chemical reactions, the factors which influence these, and the light which these relations may throw upon the enzyme actions involved.

The first step in this discussion would be an attempt to apply the equations of reaction velocity given in Chapter II to enzyme actions since enzymes manifest themselves by causing changes in such velocities. As shown in Chapter IV, the further careful study of reaction velocities has resulted in finding that a number of apparently simple chemical reactions whose changes agreed with the simple kinetic relationships, when studied more carefully showed variations which necessitated further theoretical treatment and explanation. Although the simple kinetic laws cannot therefore be applied and be expected to hold in as satisfactory a form as was thought for a considerable period of time, they should serve as a first attempt to examine the velocities of reactions as influenced by enzymes.

Before attempting to find general relations which can be used with all enzymes actions, the relations found with one enzyme will be given, and the further developments built upon these.

The reaction to be taken up in this way is the hydrolysis of sucrose by yeast sucrase. The hydrolysis of sucrose by acids was considered in Chapters II and IV.

The velocity of this reaction with sucrase has been measured repeatedly by a number of different workers. Contradictory results were obtained at times but in one of the most recent investigations[1] in which conditions were rigidly controlled, the results were put in a more satisfactory light and certain definite conclusions with regard to the kinetics of the reaction were arrived at.

In the first place, Nelson and Vosburgh applied the monomolecular velocity equations to the reaction at 37° and with different degrees of acidity (hydrogen ion concentrations). With optimum conditions of acidity, pH 4.5, it was found that the value of the velocity coefficient, *k*, increased as the reaction proceeded. With lower hydrogen ion concentrations, the value of the velocity coefficient was very nearly constant. Results were given for 24 experiments. Of these 19 gave increasing values for the velocity coefficients, 5 gave practically constant values. This shows that the reaction did not take place according to the monomolecular law, but appeared to do so under certain conditions. Where decreasing values have been found, destruction or inactivation of the sucrase during the progress of the reaction undoubtedly occurred.

With regard to the relations between the concentrations of sucrase and sucrose and the velocity of reaction, it was found that, using as a measure of comparison the time required to cause a certain change (say 40%), the velocity of hydrolysis was directly proportional to the concentration of sucrase, but not proportional to the sucrose concentration. In fact, within certain limits it was found to be independent of the sucrose concentration. With a certain sucrase solution, the velocities for the change up to 25% inversion with sucrose solutions of different concentrations, showed (for the relation between original concentration of sucrose and time for 25% inversion) increasing velocities with increase in sucrose concentration up to a concentration of

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[1] J. M. Nelson and W. C. Vosburgh, *Jour. Amer. Chem. Soc.* *39*, 790 (1917); W. C. Vosburgh, Dissertation, Columbia University, 1919.

5%, and above that constant velocity. This was found repeatedly and is evidently to be interpreted in the sense that sucrase possesses a definite capacity for accelerating the sucrose hydrolysis reaction. That is, sucrase can accelerate the hydrolysis of a definite amount of sucrose in a unit of time. Increasing the amount of sucrose above this will not increase the amount which will be hydrolyzed by the same quantity of sucrase preparation in that same length of time. This is evidently a maximum capacity and might be interpreted in several different ways. On the basis of the discussions in the preceding chapters, the most obvious explanation involves the view that the reaction takes place in several steps. In the first, combination of sucrase and sucrose may occur. In the second, this combination may decompose to form α-glucose and α-fructose. In carrying out the experimental determinations, it is always necessary, as emphasized originally by O'Sullivan and Tompson[2] to bring about equilibrium between the α- and the β-forms of the hexoses by the addition of a small quantity of alkali before determining the extent of the reaction by means of the optical rotation. If this is not done, serious errors result. The formation of this first compound between enzyme and substrate would account for the phenomenon of the saturation capacity of the sucrase observed. The practical difficulty of proving by direct experiment the existence of such a compound arises first from the fact that the enzyme is not known in an analytically pure state, secondly, the fact that the addition compound is in (colloidal) solution, and thirdly, the continuous change due to the further reaction of the enzyme-substrate complex. Even if such a compound were precipitated, the colloidal properties of the materials make it improbable that constant stoichiometrical compositions of such addition compounds can be shown to occur ordinarily.

The fact that sucrase in the presence of sucrose in solution can be heated to a higher temperature without being inactivated than in its absence is also evidence for the existence of a compound between the two.

The experimental results just outlined might also be interpreted on the basis of the formation of an adsorption compound between sucrase and sucrose. Plotting the velocity (concentration of sucrose divided by time for certain percentage hydrolyzed) against the concentration of sucrose gave curves essentially similar to adsorption curves such as have been obtained in studying the adsorption of acetic acid by charcoal.[3] Sucrase is a colloid and this relation must be considered carefully. If adsorption is the dominating factor, then hydrolysis is assumed to take place at or near the surface of the sucrase, and the effective concentration of the sucrose or the velocity of inversion might be considered a measure of the amount of sucrose adsorbed by the sucrase. The maximum velocity attained with increase in concentration of sucrose would represent upon this assumption, sucrase, saturated as regards adsorption.

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[2] C. O'Sullivan and F. W. Tompson, *J. Chem. Soc.* *57*, 834 (1890); C. S. Hudson, *Jour. Amer. Chem. Soc.* *30*, 1160 (1908).

Various mathematical expressions have been developed on the basis of the explanation of adsorption. The principle involved appears to consist essentially in replacing the concentration of the sucrose in the solution in the kinetic equation by a term involving the adsorbed sucrose. This term, according to adsorption relations, is not equal to the concentration of the sucrose in the solution, but to the numerical value of this concentration raised to some power less than unity, and very often between 0.1 and 0.5. The new kinetic equation therefore retains the same form as the simple kinetic equations given in Chapter II, except that for a monomolecular reaction, for example, the concentration of the reacting substance is replaced by the concentration raised to some power less than one, and constant within certain limits and ranges of conditions for a given reaction. This constant must be determined by means of the experimental results of the reaction itself, just as the reaction velocity constant is determined.

In its simplest terms, therefore, introducing the adsorption factor into the kinetic equations involves replacing an equation containing one arbitrary constant (which is obtained from the experimental data) by an equation containing two constants (which are obtained from the experimental data). The form of the equation makes it possible to assign a probable physical significance to the second constant as well as to the reaction velocity constant.

Nelson and Griffin[4] had shown that the reaction between sucrase and sucrose solution depended on the contact of two phases, and that the activity of the sucrase was not affected whether or not the enzyme was adsorbed to a solid, a second colloid, or distributed uniformly throughout the solution. Nelson and Vosburgh showed on the basis of their results that the velocity of inversion curve, where the concentrations of sucrose were used as abscissas, had the same general shape as adsorption curves as already suggested by Henri.[5] In addition, since their results agreed with the heterogeneous reaction view and contradicted the claim that the kinetics of sucrase action conformed to the monomolecular law for homogeneous reactions, they concluded that adsorption was one of the controlling factors in the kinetics of sucrase action.

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[3] G. C. Schmidt, *Z. physik. Chem.* *74*, 689 (1910); 77, 641 (1911).

[4] J. M. Nelson and E. G. Griffin, *Jour. Amer. Chem. Soc.* *38*, 1109 (1916).

A further relation may be developed with sucrase. It is of course known that various substances retard the actions of enzymes. Michaelis and Menten[6] showed that the action of sucrase was inhibited by the products of the reaction it accelerated. Fructose retarded the actions much more markedly than did glucose. These facts have been confirmed and extended in various directions in recent years by J. M. Nelson and his co-workers. The explanation advanced for this retardation is based upon the formation of chemical compounds of the sucrase with glucose and fructose. In this way the sucrase is removed from the sphere of action and is unable to combine with sucrose in order to cause it to react further. The suggestion may be made that adsorption compounds (in contradistinction to chemical compounds) are formed by the products of the reaction with the sucrase, thus interfering with the formation of such compounds of the latter with the sucrose. The greater retardation shown by the fructose, however, points to chemical differences as the cause of combination. It would be of interest to obtain the results of similar careful experiments with other carbohydrates, as light may possibly be thrown on the nature of the compounds with sucrase in this way.

Considerable space has been devoted to the sucrase reactions. These have been studied as extensively and as carefully as the reactions of any enzyme. The fact that the enzyme can readily be obtained, that it forms (to the eye) clear aqueous solutions (colloidal it is true), and that the chemical reaction which it accelerates has been carefully studied from various points of view, makes it a very satisfactory subject for study. The chemical nature of the active enzyme itself is unknown. On the other hand, considerable information has been obtained with regard to the mechanism of the reaction. The kinetics are fairly well known. The influences of the hydrogen ion concentration, of the sucrase concentration, of the initial sucrose concentration, of the products of the reaction, of certain neutral inorganic salts, have been studied carefully. The most satisfactory explanation of the reaction appears to involve the formation of a chemical compound of sucrase and sucrose followed by its decomposition into the hexoses.

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[5] V. Henri, *Z. physik. Chem. 51,* 19 (1905).

[6] L. Michaelis and M. L. Menten, *Biochem. Z. 49,* 333 (1913).

With regard to the explanation of adsorption for such enzyme actions, the same points may be developed as in the preceding chapters. The adsorption view alone does not appear to offer any advantages over the view of the formation of chemical compounds except that it is less specific and does not go beyond the most superficial experimental relations observed. In fact, the application of adsorption formulas does not negative the formation of addition compounds but shows only part of a possible mechanism involved in their production. Also, the view is gaining ground, as set forth in the earlier chapters, that adsorption itself is to be explained most satisfactorily on the basis of the formation of chemical compounds.

The relations outlined with sucrase will now be applied to enzyme actions in general. The underlying theory of the mechanism of its hydrolytic action on sucrose involves the formation of an intermediate addition compound of enzyme and substrate. Following the scheme of formulating reactions given in Chapter III, the following equations may be given as representing some of the possible actions in a complex mixture such as would be present in an enzyme action.

$$\left[\begin{array}{c}\text{Enzyme}\\ \text{Substrate}\\ \text{(or Products)}\\ \text{Water}\end{array}\right] \qquad \begin{array}{ll}\text{Enz.} + \text{Substr.} + \text{Water} & \text{(a)}\\ \text{Enz.} + \text{Products} + \text{Water} & \text{(b)}\\ \text{Enz. Products} + \text{Water} & \text{(c)}\\ \text{(Enz. Substr.)} + \text{Water} & \text{(d)}\end{array}$$

These equations do not show the actions of any other substances, and in fact give only the simplest changes involved. Starting with the substances of the right hand side of equation (a), if the products of equation (b) are obtained, the simplest case possible would be given. The enzyme represented as unchanged in composition would be considered to be the catalyst.

The first question which may be raised and which was not considered with sucrase, is whether starting with (b), the products of equation (a) would be obtained; in other words, whether enzyme actions are reversible. It has been found that a number of them are reversible, that certain enzymes accelerate the velocity of formation of more complex bodies from simpler ones. Questions of suitable concentration of the substances present are obviously involved and require a certain amount of experimentation to determine, but otherwise, the theoretical deduction that an enzyme, in its simplest condition of acting, accelerates both reactions involved in an equilibrium has been found to hold. Further details of this reversibility will not be given but only a list of some of the enzymes which have been found to accelerate synthetic changes will be presented. These include lipase,[7] emulsin,[8] trypsin,[9] pepsin,[10] kephirlactase,[11] maltase,[12] and oxynitrilase.[13] In some of the enzyme preparations a mixture of enzymes was present; for example, maltase from yeast extract contained at least five sucroclasts, emulsin contained at least three, etc. This served to complicate apparently some of the syntheses observed; for instance, glucose with the maltase enzyme mixtures gave isomaltose in place of the expected maltose, etc. However, these relations can be readily accounted for as being due to the mixture of enzymes present.

A question which develops directly from the idea of reversibility as dependent to a certain extent upon the concentrations of the reacting constituents has to do with the application of the kinetic equations and the limitations of the concentration terms in them. The deduction of the equations in Chapter II was based upon the law of mass action or in practical work, the law of concentration action. As deduced, the equations hold only for dilute solutions. The limit of concentration which can be used is undoubtedly different for the components of each reaction. However, this limitation must be kept in mind especially when working with concentrated solutions. Also, substituting concentration for active mass may cause complications and errors for substances of biochemical origin, where apparently simple treatments may result in modifications of properties which involve changes in the active mass of the constituent acting which is not reflected in the concentration as ordinarily measured and used.

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[7] J. H. Kastle and A. S. Loevenhart, *Am. Chem. J. 24*, 491 (1900); M. Hanriot, *C. r. 132*, 212 (1901); H. Pottevin, *Ann. Inst. Pasteur, 20*, 901 (1906), *Bull. Soc. Chim. 35*, 693 (1906); A. E. Taylor, *J. Biol. Chem. 2*, 87 (1906); M. Bodenstein and W. Dietz, *Z. Elektrochem., 12*, 605 (1906); W. Dietz, *Z. physiol. Chem. 52*, 279 (1907); A. Welter, *Z. angew. Chem. 24*, 385 (1911); *M. Krausz, Z. angew. Chem. 24*, 829 (1911); U. Lombroso, *Arch. pharm. sper. 14*, 429 (1912); K. Bournot, *Biochem. Z. 52*, 172 (1913); H. E. Armstrong and H. W. Gosney, *Proc. Roy. Soc. London (B) 88*, 176 (1914); A. Hamsik, *Z. physiol. Chem. 90*, 489 (1914).

[8] O. Emmerling, *Ber. 34*, 3811 (1901); J. H. Van't Hoff, *Sitzungsber. Kgl. Pr. akad. Wiss. Berlin, 1910*, 963; E. Bourquelot, H. Hérissey, and J. Coirre, *C. r. 157*, 732 (1913), *J. Pharm. Chim. (7) 8*, 441 (1913), E. Bourquelot (review) *J. Pharm. Chim. (7) 10*, 361, 393 (1914); G. Zemplen, *Ber. 48*, 233 (1915); E. Bourquelot and A. Aubry, *C. r. 163*, 60 (1916), *164*, 443, 521 (1917); E. Bourquelot and M. Bridel, *C. r. 168*, 253, 1016 (1919); E. Bourquelot and M. Bridel, *Ann. chim. phys. (8) 28*, 145 (1913); W. M. Bayliss, *J. Physiol. 46*, 237 (1913).

[9] A. E. Taylor, *J. Biol. Chem. 3*, 87 (1907), *5*, 381 (1909).

[10] T. B. Robertson, *J. Biol. Chem. 3*, 95 (1907), *5*, 493 (1909); T. B. Robertson and H. C. Biddle, *J. Biol. Chem. 9*, 295 (1911); F. P. Gay and T. B. Robertson, *J. Biol. Chem. 12*, 233 (1912).

[11] E. Fischer and E. F. Armstrong, *Ber. 35*, 3144 (1902).

[12] A. Croft Hill, *J. Chem. Soc. 73*, 634 (1898); *83*, 578 (1903); *Ber. 34*, 1380 (1911); O. Emmerling, *Ber. 34*, 600, 2206, 3810 (1901); E. F. Armstrong, *Proc. Roy. Soc. London (B) 76*, 592 (1905); E. Bourquelot and E. Verdon, *J. Pharm. Chim. 8*, 19 (1913).

[13] V. H. Krieble, *Jour. Amer. Chem. Soc. 37*, 2205 (1915).

To return to the general equation of enzyme action which was proposed as a consequence of the relations found with sucrase and the general theory of chemical reactions outlined in Chapter III, if equations (a) and (b) alone are considered, the enzyme would act as catalyst, being unchanged in chemical composition as a result of the reaction. Starting with the substances on the right hand side of equation (a), the substances on the right hand side of equation (b) would be obtained. As shown, the change involves two consecutive reactions. The evidence for the formation of the intermediate product in enzyme reactions, in addition to that given with sucrase, may now be presented. As pointed out in Chapter V, the formation of adsorption compounds and the greater stability toward heat of a solution of enzyme plus substrate as compared with enzyme alone, and in Chapter VI, Fischer's lock and key theory may serve as evidence. A line of proof analogous to the last was brought forward some years ago.

H. D. Dakin[14] showed that a liver lipase preparation acting upon an optically inactive mixture of dextro and lævo mandelic esters hydrolyzed the dextro component more rapidly than the lævo. His conclusions may be quoted: "The dextro- and lævo-components of the inactive ester first combine with the enzyme, but the latter is assumed to be an optically active asymmetric substance, so that the rates of combination of the enzyme with the d- and l-esters are different. The second stage of the reaction consists in the hydrolysis of the complex molecules of (enzyme + ester). Since the complex molecule (enzyme + d-ester) would not be the optical opposite of (enzyme + l-ester), the rate of change in the two cases would again be different. Judging by analogy with other reactions one might anticipate that the complex molecule which is formed with the greater velocity would be more rapidly decomposed. In the present case it would appear that the dextro component of the inactive mandelic ester combines more readily with the enzyme than the lævo-component does, and that the complex molecules (d-ester + enzyme) are hydrolyzed more rapidly than (l-ester + enzyme), so that if the hydrolysis be incomplete dextro-acid is found in solution and the residual ester is lævo-rotatory." The

[14] H. D. Dakin, *J. Physiol.* *30*, 253 (1904).

same method of reasoning may be applied to the actions of other enzymes. For example, the hydrolysis by trypsin of a number of peptides, differing only in the optical activity of the components, was found to be markedly different by Fischer and Abderhalden.[15]

If, in equation (a), the substrate is present in very great excess as compared with the enzyme, then, especially if the initial concentration of enzyme is small, a steady state may be assumed to exist in (a) in which it may be said that all of the enzyme is combined in the intermediate compound (or a constant large fraction of it is so combined). Under these conditions, if there are no side reactions or other interfering factors, the velocity of the reaction will depend upon the concentration of the intermediate addition compound, which is constant as long as sufficient excess of substrate is present. This means that as long as these conditions hold, the same absolute amount of intermediate compound is decomposed in each unit of time, or the same amount of substrate is decomposed, or the same amount of products formed. This relation, a maximum capacity of an enzyme preparation for hydrolyzing a substrate, no matter how much excess substrate may be present above a certain quantity, has been observed for a number of enzymes and taken to be evidence for the formation of such an intermediate addition compound. The following examples may be mentioned:

Sucrase : sucrose[16]
Amylase : starch[17]
Lactase : lactose[18]
Maltase : maltose[18]
Emulsin : β-glucosides[18]
Lipase : esters[19]
Urease : urea[20]

This change, when plotted, would show a linear relation between the quantity of substrate decomposed and the time, and not a logarithmic relation as required by the monomolecular reaction velocity equation.

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[15] E. Fischer and E. Abderhalden, *Z. physiol. Chem.* *46*, 52 (1905); *51*, 264 (1907).

[16] A. Brown, *J. Chem. Soc.* *81*, 373 (1902); E. Duclaux, Traité de Microbiologie, Tome II, Diastases, Toxines, et Venims, Paris (1899); L. Michaelis and M. L. Menten, *Biochem. Z.* *49*, 333 (1913); J. M. Nelson and W. C. Vosburgh, *Jour. Amer. Chem. Soc.* *39*, 790 (1917).

[17] H. T. Brown and T. A. Glendinning, *J. Chem. Soc.* *81*, 388 (1902); C. L. Evans, *J. Physiol.* *44*, 191 (1912).

[18] E. F. Armstrong, *Proc. Roy. Soc. London (B)* *73*, 500 (1914).

[19] H. C. Bradley, *J. Biol. Chem.* *8*, 251 (1910); G. Peirce, *Jour. Amer. Chem. Soc.* *32*, 1517 (1910); K. G. Falk and K. Sugiura, *Jour. Amer. Chem. Soc.* *37*, 217 (1915).

[20] D. D. Van Slyke and G. E. Cullen, *J. Biol. Chem.* *19*, 141 (1914).

In a number of these cases, the amount of action was found to be proportional to the enzyme concentration (with large excess of substrate present).

The evidence may be considered sufficient to prove the formation of intermediate addition compounds with the enzyme in reactions which are accelerated by enzyme preparations.

If the substrate is not present in great excess, and if there is no steady state existing in either of the two reactions, then the kinetics would be represented by the equations for consecutive reactions which were given in Chapter II. These equations are very complex and require further assumptions and simplification in order to be used in any given case. In view of the experimental complications involved in enzyme actions, some of which will be taken up presently, it does not seem to be worth while to enter into the details of this question at present.

If one of these reactions takes place much more slowly than the other, the one with the smaller velocity will be the one to be observed experimentally. It may be possible to alter the conditions with certain enzymes so that either one of the reactions would be studied independently.

Another factor must be considered in this connection. If a complex substrate is being decomposed, a number of products may be formed (as in the breaking down of a protein or a starch). The second stage of the reaction would then consist of a number of consecutive reactions. The extent of the changes in the substrate might then give different results depending upon whether the decomposition of the substrate or the formation of different sets of products was being studied. This was pointed out in Chapter VII, in which the results of Sherman and his co-workers were given, which showed that somewhat different results were obtained in studying the action of amylase upon starch, depending upon whether the amyloclastic (starch-splitting) or saccharogenic (sugar-forming) properties were followed. Some apparently contradictory results recorded in the literature might be accounted for in this way.

The conditions in any one series of experiments must be kept constant. In order to obtain comparable results for kinetic studies, it is advisable to work at the optimum hydrogen ion concentration of the reaction. Two investigations give some suggestive data in this connection, indicating perhaps a possible mass action effect in the enzymic hydrolysis of proteins. Frankel[21] found that when papain acted upon gelatine or egg albumin (optimum pH 5.0), if the reaction initially was more acid or more basic than the optimum, it gradually tended to approach the optimum hydrogen ion concentration. In other words, the hydrolysis may have taken place in different ways in the two cases, in the first, a greater number of amino groups than of acid groups were formed, in the second, a greater number of acid than of amino. If the reaction started at pH 5.0, the basic and acid groups would be formed in a ratio which kept the acidity constant. Essentially the same phenomenon was observed by Long and Hull[22] in the action of trypsin on fibrin or casein. On the other hand, this change may have been due to the properties of the aminoacid or similar substance split out by the action of the enzyme. The aminoacid may have an isoelectric point in the neighborhood of pH 5. and, exerting a buffer action, tend to bring the acidity nearer to that point.

So far, the mechanism of the action has been assumed to be simple and straightforward. Some of the possible complicating factors must now be considered. In the preliminary outline of sucrase action, it was pointed out that the products of the reaction may interfere by combining with the enzyme and so removing it from the sphere of action. This possibility is indicated in equation (c) of the general formulation. It can readily be studied by adding some of the products which would be formed in the reaction at the beginning of the action. Combination of enzyme and products has been found to occur with a number of the enzymes. In addition to sucrase to which reference has been made, the same relation was found for amylase and maltose,[23] pepsin and peptone (or albumose)[24] and doubtless others. Related to these actions is the influence of inhibiting substances on enzymes explained most satisfactorily as a combination of the inhibiting substance with the enzyme, thereby preventing the combination of the enzyme with its substrate and the consequent transformation of the latter. The antitryptic action of serum albumin was explained in this way.[25] This relation is mentioned here as indicating one of the possibilities of retarding action which must be watched for in studying the kinetics of enzyme actions. The question may be raised whether the anti-enzymes which are spoken of at times do not exert their influence in this way by combining with the enzyme and being of such nature themselves that they are not decomposed.

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[21] E. M. Frankel, *J. Biol. Chem.* *31*, 201 (1917).

[22] J. H. Long and M. Hull, *Jour. Amer. Chem. Soc.* *39*, 1051 (1917).

[23] A. Wohl and E. Glimm, *Biochem. Z.* *27*, 349 (1910); G. McGuire and K. G. Falk, *J. Gen. Physiol.* *2*, 224 (1920).

[24] J. H. Northrop, *J. Gen. Physiol.* *2*, 471 (1920).

[25] S. G. Hedin, *Z. physiol. Chem.* *52*, 412 (1907).

In any event, the combination of enzyme with products of the reaction, which has been made to appear probable in a number of cases, would result in a progressive slowing up of the reaction. It would show its action on the velocity determinations and calculations in the same way as if the enzyme were destroyed. Actually, an inactivation, probably reversible in the first case, irreversible in the second, takes place with both.

Another possible complicating factor in following the kinetics and studying the mechanism of enzyme actions has to do with the substrate. Many enzymes act upon substrates of very complex nature. It would not be surprising, therefore, if such substrates would have to be placed under definite conditions in order to obtain optimum, or perhaps even at times, marked, hydrolytic actions. This question was taken up with the proteins in a number of investigations. For example, Northrop [26] considered that there is an optimum zone of hydrogen ion concentration for the combination of pepsin and protein corresponding to an optimum of digestion. He suggested that the pepsin combined largely or entirely with ionized protein. Long and Hull [27] had come to similar conclusions for the action of trypsin, suggesting that it was the combination of enzyme plus substrate rather than the enzyme alone which was affected by the reaction. Their results showing that with the same trypsin, different optimum hydrogen ion concentrations were obtained with different proteins have already been referred to. Ringer [28] also has emphasized the importance of the substrate in the action of pepsin. He, however, considered that the optimum action depended upon and corresponded to a maximum swelling (hydration) of the protein.

These investigations all point to the importance of the complex substrate in enzyme actions. Chemical changes are evidently involved here as in the changes which enzymes undergo in becoming inactivated. It appears to be perfectly proper therefore to say that a substrate is inactivated when, by a simple change in conditions, it is found to be no longer in a state to be acted upon by an enzyme. From this point of view, such differences would present only a phase of the general phenomenon of specificity.

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[26] J. H. Northrop, *J. Gen. Physiol.* *2*, 113 (1919).

[27] J. H. Long and M. Hull, *Jour. Amer. Chem. Soc.* *39*, 1051 (1917).

[28] W. E. Ringer, *Z. physiol. Chem.* *95*, 195 (1915) ; *Kolloid. Z.* *19*, 253 (1916).

There is another way in which the substrate can exert an influence. This may be illustrated by the action of a castor bean lipase preparation on a number of different esters,[29] and also its action in the presence of different alcohols on an ester.[30] The results showed that retardation on the hydrolysis of ethyl butyrate was found with methyl and ethyl alcohols, the amounts of the retardation increasing with the concentration. On the other hand glycerin tested in the same way as the alcohols caused no retardation even up to a concentration of 25%.[31] The hydrolytic action of the lipase preparation was then tested on varying concentrations of a number of different esters. A certain complication was introduced by the fact that a mixture of ester-hydrolyzing enzymes was undoubtedly present. Starting with no ester and increasing the quantity up to molar concentration, it was found that with methyl acetate increasing actions were obtained up to 0.2-0.5 molar and then decreasing actions; with ethyl acetate, the same, beginning to decrease at a smaller concentration; with ethyl butyrate, increasing actions up to 0.2 molar reaching a value double that of the ethyl acetate, and then remaining constant for the greater concentrations (possibly because the limit of solubility of the ester had been reached); and with glyceryl triacetate, continually increasing actions with increasing concentrations, with little of the marked retardations shown by the other esters.

These results justify the extension of the explanation advanced for the action of the simple alcohols suggested in Chapter V to the action exerted by the simple esters; i.e., the ester causing a change, possibly accompanied by precipitation or coagulation of substances in the course of which the active lipase material is partially or wholly removed from the sphere of action. Methyl acetate and ethyl acetate showed least increase in activity with increasing concentration of ester and therefore the greatest inhibiting action. Glyceryl triacetate showed the greatest increase in activity with increasing concentration of ester and therefore the smallest inhibiting action. These results are exactly similar to those obtained with methyl and ethyl alcohols and glycerin. That the actions are not controlled entirely by the alcohol radicals is apparent from the fact that with glyceryl triacetate even the dilute solutions did not show a proportionality between the amount of ester and the action. To continue this view further, it is probable that the fats, such as glycerides of fatty acids of high molecular weight, doubtless exert practically no inhibiting action. The rate of their hydrolysis would then depend upon the possibility of dissolving the fat or of bringing it in direct contact with the enzyme. To how great an extent accelerators such as bile salts act in this way does not appear to be quite certain as yet.

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[29] K. G. Falk, *Jour. Amer. Chem. Soc. 35,* 1904 (1913).

[30] K. G. Falk, *Jour. Amer. Chem. Soc. 35,* 616 (1913).

[31] Cf. also H. E. Armstrong and E. Ormerod, *Proc. Roy. Soc. London (B) 78,* 376, (1906).

These results appear to account for part, at any rate, of the specific actions of the lipase on different esters. In these results, it must be remembered, the hydrogen ion concentration was not fixed. However, many of these and analogous experiments on specificities are incomplete in this way. The explanation and analogy given may therefore account for them. However, for more complete and satisfactory conclusions to be reached it is necessary to consider the hydrogen ion concentrations, possible salt actions, etc.

A similar or possibly analogous action of substrate on enzyme must be looked out for in every enzyme action. The effect may be small or even absent, or again, as with lipase, it may be quite marked.

These two possible effects or actions of substrate on enzyme are typified by equation (d) in the general reaction. The enzyme-substrate is indicated in parentheses to show that there is no compound as such formed and remaining as a product, but only to indicate that the substrate acts on the enzyme in some way.

The consideration of the mechanism of enzyme actions shows the difficulty of applying simple kinetic equations to the reactions. Only in special cases do the results show that certain relations hold. It may be mentioned that Bayliss,[32] following Bredig,[33] pointed out that the best means of comparing the relative velocities, without assuming the application of some law of reaction velocity, was to use the time required to effect a definite change as a measure of the reaction velocity.

A number of attempts have been made to derive general equations for enzyme actions involving relations similar to those given in equations (a), (b), and (c), of the general formulation. Unfortunately, in most of these, expressions were obtained which contained two or more constants which had to be obtained from the experimental results to which they were later applied. These expressions obviously were found to hold more satisfactorily than the more usual one-constant expression, but did not prove that the relations postulated were correct. Also, at times, new relations would be developed and conclusions drawn which involved assumptions which were sometimes stated and sometimes omitted. It seems hardly necessary to enter into any of these deductions and theories. None has found universal acceptance, and while some have been used for special investigations, the limitations as a rule have early been recognized.

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[32] W. M. Bayliss, *Proc. Roy. Soc. London (B) 84,* 87 (1911).

[33] G. Bredig, *Ergeb. Physiol. 1,* 134 (1902).

At the same time, some deductions have been made and kinetic equations developed in which the complex nature of the problem was recognized and the limitations of the equations, as well as the assumptions made, clearly stated. The most recent of these was published by Northrop [34] in connection with the study of the action of pepsin on albumin. Here, the relations were developed essentially with the use of the three equations (a), (b), and (c) of the form given above. In the deductions, certain assumptions were made in order to make possible the application of the kinetic equations to the experimental results, but in every case, the experimental justification for these assumptions was pointed out. One of the interesting developments is the fact that under certain conditions (concentration of products large with respect to pepsin, and concentration of substrate relatively constant) the equation deduced simplified to Schütz's empirical rule [35] which had been found to hold for a number of enzyme actions under certain conditions, that the amount of action was proportional to the square root of the quantity of enzyme. The equation of Northrop showing this, is similar to one derived by Arrhenius [36] to show a chemical analogue to Schütz's rule, which he found was true of the hydrolysis of ethyl acetate by ammonia in aqueous solution.

The detailed developments given by Northrop will not be attempted here, partly because his equations, strictly speaking, apply only to the reaction which he was studying. His work, however, will serve to show the complexity of the problem of the kinetics of enzyme action as well as the methods which may be used to throw light on the questions involved, and for these reasons deserves careful study.

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[34] J. H. Northrop, *J. Gen. Physiol.* 2, 471 (1920).
[35] E. Schütz, *Z. physiol. Chem.* 9, 577 (1885).
[36] S. Arrhenius, "Quantitative Laws of Biological Chemistry," 1915, p. 41.

IX.—Uses and Applications of Enzymes

In this chapter an attempt will be made to indicate some of the circumstances under which enzymes and enzyme actions touch upon the problems and manifestations of things met with in the ordinary course of affairs. The utilitarian aspects rather than the purely scientific (or, as some would have it, non-utilitarian) side of enzyme actions will be emphasized. At the same time it will be seen that enzyme actions show clearly the close relationship which exists between the scientific and the practical aspects of chemical phenomena.

Enzymes have been defined as catalysts produced by living matter. It is difficult to give a satisfactorily rigid definition of living matter, but the evidence available at the present time indicates that the chemical reactions which take place during life processes obey the same laws as chemical reactions unconnected with such processes. The evidence is only fragmentary at the present time, it is true, but this may be due to the complexity of the substances involved and the lack of accurate knowledge concerning their chemical nature. The complexity of these substances also involves the view that they may react in a number of different ways. Also, none, or very few, of them may be said to be (thermodynamically) stable. That is to say, given sufficient time, practically all of them would decompose to give finally a limited number of stable products in appreciable quantity, together with traces of a greater number of products.

The chemical reactions which take place in living matter must be influenced in such a way that the products formed are essential to the life process, if life is to continue. The actions of enzymes would therefore consist in favoring or accelerating those reactions which are required in the life process and making possible its continuance. On the basis of the general theory of chemical reactions referred to in the earlier chapters of this book, this would mean that the complex addition or intermediate compound formed by the reacting substances plus enzyme catalyst reacts in certain more or less definite ways. In this way certain products would be formed with velocities sufficiently great to make other possible products, as well as products zyme catalyst, ordinarily negligible in quantity. The function of the enzyme is, therefore, to favor the production in living matter of certain products which might otherwise be formed in only small amounts. The question may be raised whether the enzyme catalyst can start a reaction or only modify the velocity of a reaction which can take place in its absence but much more slowly. This question has been much discussed. Following certain thermodynamical considerations, the catalyst only modifies the velocity of a reaction; but practically speaking, a catalyst may increase the velocity of a reaction which proceeds almost infinitely slowly in its absence, sufficiently to make the reaction of practical use.

Enzymes, or preparations obtained from biological material, have been observed to accelerate a great number of reactions in vitro, similar to the reactions occurring in vivo. It has therefore been assumed that these same enzymes accelerate these reactions in living matter. This assumption can hardly be questioned. The general considerations of chemical reactions indicate the part played by the enzyme. Briefly, the presence of the enzymes favors the changes in certain directions over those in other directions, possibly by means of the mechanism already outlined. In view of the manifold possibilities of chemical reactions with such complex molecules as are generally present, it may be asked whether all chemical reactions of living matter may not be influenced by enzymes, and further whether enzymes are not the essential constituents of living matter in bringing about the chemical reactions which accompany life. This is perhaps not the place to enter into a discussion of this question. It is mentioned because of the importance which enzymes have already reached in biochemical studies and is therefore suggestive of their still greater and widespread significance. In any event, so many living processes, whether of animal, plant, bacterial, etc., nature, have been found to be connected with enzyme actions, that these actions show at present, without considering possible future developments, a sufficiently inclusive field to illustrate the practical significance of the study of the uses of enzymes and their actions.

It has already been indicated that enzyme actions are present in all living matter and are connected with the chemical changes occurring there. This includes all the phenomena accompanying growth, including all metabolic and catabolic changes. Enzymes are only concerned with systems in process of chemical change. Evidence has been accumulating to show that an enzyme in a definite preparation is capable of accelerating a more or less definite reaction. This reaction may refer to a change taking place in one substance only, or it may refer to analogous changes occurring with a group of related substances (such as the hydrolysis of esters). If a given preparation can accelerate the velocities of reaction of two different groups of substances, then it is customary to assign two distinct enzymic properties or enzymes, one for each group of reactions, to the preparation. This property of specificity spoken of in Chapter VI will be referred to again in Chapter X. It is of interest and importance in this connection as it is one of the most significant of the enzyme properties and is made use of in almost every connection in which enzymes and enzyme reactions are employed.

The uses and applications of enzymes may be divided roughly into the following groups:

Industrial applications of enzymes.
Enzymes of metabolism and catabolism.
Enzymes in plant growth.
Bacterial enzymes.
Enzymes in laboratory work.

This list does not pretend in any way to be complete. It will serve to indicate the manifold possibilities of the utilitarian side of enzyme actions. Obviously it would lead too far to enter into any one of these groups to any extent here. Monographs might be written on any one of them. At the same time it is true that the work with them has been descriptive to a great extent in the past. The enzymes which have been studied carefully, such as some of those involved in the chemical reactions of metabolism, have been included in the earlier chapters.

With regard to the industrial applications of enzymes, the fermentation industries represent perhaps the most important group at the present time. A number of the problems connected with alcoholic fermentation have been described in some detail by A. Harden in "Alcohol Fermentation."[1] The discovery by Buchner[2] that the liquid obtained from yeast cells by the use of high pressure after grinding with sand, in the complete absence of cells was capable of forming carbon dioxide and alcohol from sugars, showed that the action was due to an enzyme which he called zymase and was not directly dependent upon the life process. Reference may be made to the monograph of Harden for the various directions these studies have taken. Only a few points

[1] Monographs on Biochemistry, 1911.

[2] E. Buchner, *Ber.* *30*, 117, 1110 (1897).

can be mentioned here. The yeast juice contains a mixture of enzymes which can give rise to a number of different products. The zymase which converts hexoses into alcohol and carbon dioxide is of course the most important enzyme or group of enzymes. Pasteur[3] showed in 1860 that glycerin was formed by the action of yeast on invert sugar. In view of the glycerin shortage in recent years, attempts were made to apply the fermentation process to this purpose. This was successfully accomplished[4] and glycerin will undoubtedly be produced in this way to a certain, doubtless increasing, extent, in the future. The principle involved may be outlined briefly as follows: Glycerin is formed to a small extent, perhaps 3%, from sugar in the ordinary yeast fermentation. If the mixture is made more alkaline by the addition of various substances such as sodium bicarbonate, disodium phosphate, etc., the amount of glycerin formed is increased up to 12-13% of the sugar decomposed. On the other hand, this more alkaline mixture is a good breeding medium for acid forming bacteria which would pollute the glycerin. The addition of a sulfite, either sodium or calcium, acts as poison for the lactic acid bacteria and others, but does not affect the yeast cells, and keeps the mixture sufficiently alkaline. The yield of glycerin under these conditions is 23-36.7% of the sugar. Very nearly an equivalent amount of acetaldehyde is produced, the reaction equation apparently being as follows:

$$C_6H_{12}O_6 = C_3H_8O_3 + C_2H_4O + CO_2$$

The acetaldehyde combines with the sulfite and can be recovered from this. The increase in sulfite concentration decreases the amounts of alcohol and carbon dioxide which are formed as in the ordinary alcoholic fermentation, until with a suitable concentration, this reaction becomes of minor importance. The course of these reactions has not been cleared up satisfactorily with regard to the formation of the various products.

The studies of H. Euler and his associates on yeast fermentation which have been appearing regularly in the past years are yielding valuable information on a number of the enzyme properties and enzyme actions.

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[3] L. Pasteur, *Ann. Chim. Phys. (3) 58,* 347 (1860); Cf. also E. Buchner and J. Meisenheimer, *Ber. 39,* 3201 (1906).

[4] W. Connstein and K. Lüdecke, *Ber. 52,* 1385 (1919); C. Neuberg and E. Reinfurth, *Biochem. Z. 89,* 365 (1918); *92,* 234 (1918); *Ber. 52,* 1677 (1919); E. Zerner, *Ber. 53,* 325 (1920); Cf. also A. R. Ling, *J. Soc. Chem. Ind. 38,* 175R (1919) for process developed by J. R. Eoff, W. V. Linder, and G. F. Beyer for the Treasury Department of the United States.

The recent observations of Harden[5] that zymase and dried yeast which have been inactivated by washing can be activated again by the addition of potassium phosphate and a pyruvate or acetaldehyde, and also that a specific difference in relation to alcoholic fermentation exists between sodium on the one hand and potassium and ammonium on the other, involve interesting possibilities.

Among the other products obtained in fermentation by yeast are succinic acid, acetic acid, formic acid, esters, fusel oil, etc. Fusel oil consists of a mixture of some of the higher alcohols and probably aldehydes. F. Ehrlich[6] showed that these alcohols were formed from aminoacids present according to the following equation:

$$RCHNH_2CO_2H + H_2O = RCH_2OH + CO_2 + NH_3.$$

Ehrlich was not prepared to decide whether the fusel oil formation was a purely enzymic action or whether it was also connected with the life process of the yeast.

The work of Dernby[7] on the autolysis of yeast has been referred to in another connection but is of special significance here. Dernby studied the optimum hydrogen ion concentrations for the pepsin, tryptase, and ereptase of yeast and the changes in action with changes in acidity. The optimum pH values found were 4 for pepsin, 7.0 for tryptase, 7.8 for ereptase. Plotting the curves for amounts of actions and different pH values for the three enzymes, in order to obtain the maximum autolysis at a constant hydrogen ion concentration, he showed that the value for pH should be 6, between the optima for pepsin and the other enzymes where all can exert their actions.

Dernby's work on the autolysis of animal tissues gave similar results.[8] For example, it explains the fact that a piece of tissue does not autolyze in alkaline solution, but if placed in acid first (for pepsin action) and then in alkali (for trypsin and erepsin action) rapid autolysis occurs.

The use of various amylase preparations industrially is widespread. It is only necessary here to refer to takadiastase, pancreatin, and malt diastase which are all official pharmaceutical preparations.

The production in recent years of acetone by fermentation is of interest as showing the industrial possibility of such a reaction[9] even if it may not be able to compete at the present time under normal conditions with the older processes. Two investigations taking up the study of this reaction were published recently. The biochemistry of *Bacillus Acetoethylicum* with reference to the formation of acetone from starch or glucose was studied.[10] The optimum condition of the medium for growth was found to be pH 8.0-9.0; for fermentation 6.0-8.0. A number of different sugars were found to be fermentable using calcium carbonate, and peptone (as source for nitrogen), 43° was found to be the most satisfactory temperature. Formic acid and ethyl, propyl, and butyl alcohols were also formed. The general conditions for carrying out the process so that acetone and ethyl alcohol were the main products and a semicontinuous method for carrying on the fermentation were described.[11] Recently, a similar study of the acetone and butyl alcohol fermentation of starch by *Bacillus Granulobacter Pectinovorum* (which was used commercially) was published.[12]

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[5] A. Harden, *Biochem. J. 11,* 64 (1917).

[6] Cf. F. Ehrlich, *Biochem. Z. 2,* 52 (1906) for a review and summary of this work.

[7] K. G. Dernby, *Biochem. Z. 81,* 109 (1917).

[8] K. G. Dernby, *J. Biol. Chem. 35,* 179 (1918).

An interesting investigation on the acid fermentation of xylose by bacteria found in fresh silage, sauerkraut, manure, and in certain soils was published recently.[13] The main products obtained were acetic acid and racemic lactic acid in a proportion of 43 to 57 parts by weight. The best conditions for this action were determined.

Additional studies of similar nature might be given but would serve no useful purpose here. A comparison of the conditions used and the results obtained in various fermentations undoubtedly would give conclusions of importance and value in classifying and carrying out the desired chemical reactions.

It does not appear necessary to enter further into the industrial applications of enzymes. Reference may be made to the larger volumes on enzyme actions such as the treatise on "Biochemical Catalysts in Life and Industry"[14] which gives a satisfactory and complete account of proteoclastic enzymes.

Some general principles involved in these actions may, however,

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20, 874 (1906); F. Schardinger, *Wien klin. Woch. 17*, 207 (1904); *Centr. Bakteriol., 2te Abt. 14*, 772 (1905). Two processes have been used on a commercial scale: F. Bayer and Co. D. R. P. 283,107, July, 1913; 291,162, Jan. 1914; Brit. Pat. No. 14371, June, 1914; K. Delbrück and K. Meisenburg, U. S. Pat. 1,169,321; A. Fernbach and E. H. Strange, U. S. Pat. 1,044,368; 1,044,446; 1,044,447.

[10] J. H. Northrop, L. H. Ashe, and J. K. Senior, *J. Biol. Chem. 39*, 1 (1919).

[11] J. H. Northrop, L. H. Ashe, and R. R. Morgan, *J. Ind. Eng. Chem. 11*, 723 (1919).

[12] H. B. Speakman, *J. Biol. Chem. 41*, 319 (1920).

[13] E. B. Fred, W. H. Peterson, and A. Davenport, *J. Biol. Chem. 39*, 347 (1919).

[14] By Jean Effront, translated by S. C. Prescott, 1917.

be stated. The advantages in the industrial use of enzyme actions may include the comparatively low temperatures at which the reactions take place, the greater or less specificity of the actions making it possible to control the products, and the probable low cost of the materials used. The disadvantage is the length of time which may be needed to carry out the reaction. This disadvantage can in part be met by a study of the optimum conditions of the reaction in question, application of such principles as the law of mass action as involved in the kinetics of the reaction, and a search for substances showing accelerating actions.

Another possible application of enzyme actions in industry involves synthesis in place of decomposition. This possibility does not appear to have been considered to any extent as yet. Because of the comparatively small amount of data available in this field, it would be necessary to carry on considerable pioneering work before any statement of its probable success could be ventured.

It would also appear that a careful study of the oxidizing enzymes might show some which would be of practical use.

The use of enzymes as reagents in laboratory work has been begun and promises to be of considerable value. The most striking example of their usefulness in this way is to be found with the enzyme urease. The specificity of the soy bean urease had been pointed out clearly by Armstrong and Horton.[15] For example, it was shown to hydrolyze urea and not substituted ureas such as methylurea. The accurate estimation of urea in animal fluids such as blood and urine had always been a most troublesome operation. Marshall[16] showed that the use of soy bean urease overcame all the obstacles in a very simple manner; that after the removal of the ammonia which may be present initially, addition of ground soy beans or an extract of soy beans hydrolyzed the urea quantitatively so that the determination of the ammonia formed from the urea either by titration with the use of a suitable indicator or by an aeration procedure, was a simple matter. Following Marshall, a number of investigators improved the carrying out of the method in minor details. At the present time, with the use of the enzyme urease, the determination of urea has become an operation which can be carried out comparatively simply and with a satisfactory degree of accuracy.

The use of certain of the sugar hydrolyzing enzymes has been suggested and adopted in analytical work. Kjeldahl[17] and also O'Sullivan and O'Sullivan and Tompson[18] proposed to use sucrase for the determination of sucrose and Ling and Baker[19] as well as Ogilvie[20] used it for sucrose in cane and beet products. The further development has made it possible to estimate the trisaccharide raffinose by the suitable choice of enzyme materials. The method was developed by Hudson[21] and was outlined as follows:

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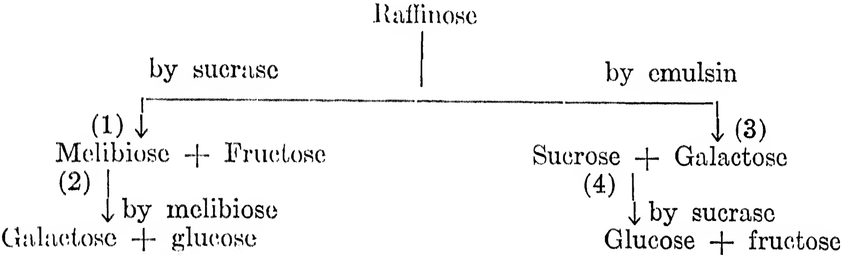
[15] H. E. Armstrong and E. Horton, *Proc. Roy. Soc. London (B) 85*, 109 (1912).
[16] E. K. Marshall, Jr., *J. Biol. Chem. 17*, 351 (1914).



Raffinose on treatment with mineral acids yields equal molecules of glucose, fructose, and galactose. With sucrase alone it yields melibiose and fructose; with emulsin alone, sucrose and galactose. From top yeast an extract rich in sucrase but containing no melibiose may be prepared, from bottom fermentation yeast, an extract may be prepared containing both enzymes. In carrying out an estimation of raffinose,[22] top yeast extract (sucrase) was used first. This inverted all the sucrose present and converted the raffinose into melibiose and fructose (reaction (1)). Using bottom yeast extract then, the change in rotation showed the hydrolysis of the melibiose (reaction (2)) and permitted of the calculation of the raffinose. If melibiose were present originally it would interfere with the determination, but in some cases this can be corrected for with the original solution because melibiose reduces Fehling solution and raffinose does not.

Parenthetically it may be remarked that this scheme of analysis is similar to that used with synthetic, optically active tri- and tetrapeptides with different trypsins to find the point of attack by the enzyme by following the change in rotation.[23] The following example may be given:

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[17] J. Kjeldahl, *Compt. rend. Carlsberg Laboratoire, Copenhagen, 1,* 189 (18881).

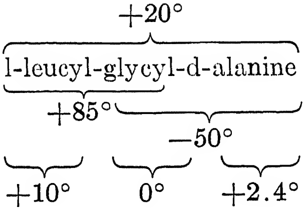
[18] C. O'Sullivan, *J. Chem. Soc. 49,* 58 (1886); C. O'Sullivan and F. W. Tompson, *Ibid. 59,* 46 (1891).

[19] A. R. Ling and J. L. Baker, *J. Soc. Chem. Ind. 17,* 111 (1898).

[20] J. P. Ogilvie, *J. Soc. Chem. Ind. 30,* 62 (1911); *Int. Sugar J. 14,* 89 (1912).

[21] C. S. Hudson, *Jour. Amer. Chem. Soc. 36,* 1566 (1914).

[22] C. S. Hudson and T. S. Harding, *Jour. Amer. Chem. Soc. 37,* 2193 (1915).



Following the change in rotation on enzymic hydrolysis should show between which groupings hydrolysis occurred if the reaction took place in steps. With pancreatic extract and intestinal extract, rotation increased first to about 40° (or l-leucyl-glycine and d-alanine were formed); later the rotation decreased (hydrolysis of l-leucyl-glycine). Glycyl-d-alanine apparently was not formed at all with these reagents (if enzyme preparations may be so termed). With yeast extract, on the other hand, rotation decreased, showing that l-leucine was first separated. A number of similar examples were given, but not sufficient to enable generalizations to be drawn.

In any event, these two series of reactions with sucroclastic and proteoclastic enzymes indicate interesting and perhaps useful applications of enzyme actions.

It is possible to use certain enzymes as reagents for testing for individual substances. For example, the fact that emulsin (obtained perhaps best from sweet almonds) hydrolyzes β-glucosides and not α-, and that maltase hydrolyzes α-glucosides and not β-, makes it possible with these enzyme preparations to determine whether in a doubtful case, an α- or a β-glucoside is present. A limitation is involved here in that only the dextro forms are hydrolyzed by these enzymes, not the lævo. Also in a recent investigation[24] it appeared probable that potato juice contained in the neighborhood of one per cent sucrose (or possibly raffinose), since it was acted on in this proportion by a yeast sucrase preparation which did not hydrolyze maltose.

These examples show the lines which the laboratory uses of enzyme preparations have followed in recent years, and make it evident that considerable possibilities for further development exist here.

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[23] E. Abderhalden and A. H. Koelker, *Z. physiol. Chem.* *51*, 294 (1907); *54*, 363 (1908); *55*, 416 (1908).

[24] G. McGuire and K. G. Falk, *J. Gen. Physiol.* *2*, 215 (1920).

X.—Present Status of the Enzyme Problem

In discussing the present status of the enzyme problem, the views presented will be those of a chemist who has worked on certain phases of the question, and whose personal outlook, inclinations, associations, and opportunities for study, will color his opinions to a certain extent. Most stress will therefore be placed on the chemical features of the problem. The views of a physiologist, of a botanist, or of a biologist, in discussing the same questions might well lead to entirely new viewpoints and emphasize different aspects of the subject. Bearing in mind the limitations indicated, a brief summary of some of the questions will be presented.

A review of the topics treated in the earlier portions of this book may make the point of view clearer. In the first chapter the general problem of enzymes and enzyme actions was outlined and the two general lines of investigation stated which appear at the present time to offer the most promise. These include the careful study of the kinetics of enzyme actions and the study of enzymes as substances possessing definite chemical structures or configurations. The purpose of this monograph is to point out the progress which has been made in these lines of investigation. This method of treatment implies that enzymes are to be considered as chemical substances which obey the laws of chemistry, and whose actions are explainable by the theories in vogue. In short, enzymes and enzyme actions form an integral and active part of chemical science, falling in line with present theories, suggesting new hypotheses, and together with other topics and fields of chemistry, correlating facts and relations.

In order to bring out these views, some of the general relations and theories of chemistry which are most closely related, and which apparently may be applied to enzyme actions directly, are outlined in the first three chapters. Chapter I also includes some of the more recent views of chemical structure, in Chapter II are stated the elementary equations of chemical kinetics, since enzymes manifest their actions by changes in velocities of chemical reactions, with special emphasis placed on the limitations of the deductions, while in Chapter III a general theory of chemical reactions, including catalytic reactions, is given as a necessary foundation for the further developments and classifications. The theoretical considerations given in these three chapters at first sight are not needed in the usual study of enzyme actions, but if the latter are to be treated as, and made part of, one of the general problems of chemistry, they must be included in some form. Generally, enzyme actions are spoken of as chemical actions and the subject disposed of in this way without stating what is meant by this all-inclusive generalization. It appears to be advisable for further progress to be more specific even at the risk of finding the results which may be useful somewhat meagre in quantity, and the theoretical developments which have been given not always sound. Following the lines of reasoning indicated, in the fourth chapter some of the properties, relations, and theories involved in some of the simpler chemical reactions of use in enzyme studies are outlined. It is shown that, while much has been done in the way of systematizing the relations and many questionable points have been cleared up, a completely satisfactory explanation and theory of even the simplest and most carefully studied of these reactions is not at hand.

The more direct description of enzymes and enzyme actions is then taken up. Physical properties common to enzyme preparations are described in Chapter V. The views on adsorption as possibly the main feature of enzyme actions are given. The relation between chemical structures and adsorption are discussed and the view upheld that the chemical structure is the predominating influence in these actions. The sixth chapter treats of chemical properties common to enzyme preparations. The distinction between physical and chemical properties is arbitrary and artificial to some extent and is made mainly as a matter of convenience. The main chemical property considered is that connected with the acidity of the medium or mixture in which the enzyme action takes place. A number of questions connected with this property are also taken up. Interesting parallelisms between the behaviors of indicators and of enzymes are pointed out. The specificity of enzyme actions is also considered briefly. The probable chemical structures of some enzymes and the methods which have been used to obtain the purest and most satisfactory preparations are described in Chapter VII. These methods indicate also the manner in which new enzyme problems may be taken up in as far as obtaining the necessary material is concerned. The mechanism of enzyme actions, based fundamentally on the kinetic equations given in Chapter II, is discussed in Chapter VIII. The complicated nature of the reactions, the underlying principles involved, and some of the attempts at solving the relations are described. A careful study of some of the work which is outlined in this chapter taken in connection with the contents of Chapters II and IV will serve to show the difficulties which are encountered in applying exact kinetic equations to the experimental work with enzymes and the necessity for making clear the various assumptions which are introduced in such measurements and calculations. A few of the industrial and laboratory uses and applications of enzymes are outlined in Chapter IX.

The colloidal property has been assumed in the past to be to a certain extent characteristic of enzymes. In the present treatment, the colloidal property has been ignored or at least relegated to a subordinate position in considering the actions. The enzyme substances are assumed to behave on chemical treatment just as other substances behave. The exact state of a substance, whether gaseous, liquid, solid, or in solution, will naturally modify the relations observed, but fundamentally the chemical reactions of a substance are based upon its chemical properties. There appears to be no valid reason to separate colloidal substances and to attempt to develop a new point of view in order to discuss the chemical reactions of such substances. Obviously, with a different set of substances, or with the same set of substances in a somewhat different state, experimental work will bring out some heretofore unknown or ignored property. The view is gaining ground rapidly that the attempts to develop a colloid chemistry distinct from ordinary chemistry have failed and that the most satisfactory point of view lies in considering colloid chemistry as a part of chemistry, at the same time including those portions of physics which apply more directly to the phenomena. As stated already, the colloidal property of most enzyme preparations may be due to the fact that these are obtained from biological material. Attempts to obtain simpler enzyme bodies from the complex materials have resulted in all cases in destruction of the enzyme properties. The enzyme property, however, is not necessarily connected with the colloidal property. It should therefore be possible to obtain the former in some way even from biological material, in a crystalloidal or dialyzable state. This view, as well as other views to be outlined presently, may perhaps be thought to be too simple for the complex conditions which are met with in enzyme work. There is no intention of ignoring the great experimental difficulties which are met with in enzyme work, and the very unsatisfactory material (chemically speaking) which must so frequently be handled. On the other hand, it is desired to throw out as far as possible complex theories of actions and to go back to definite chemical principles. In going back to such principles and relations it is not meant that the phenomena can be treated at present as simply and as satisfactorily either from the experimental or the theoretical side as those phenomena which have been under investigation for hundreds of years. It is not known whether the presentation of such a point of view is successful in the present instance. Whether it is or not, it is the belief of the writer that this is the direction in which progress can be achieved.

The study of the chemical structures of enzymes is part of the study of the chemical structures of substances of biochemical origin. In isolating such substances from living matter, changes are frequently brought about by the reagents or treatments employed. Especially with substances of large molecular weight, colloidal substances, and those possessing optical activity, would changes be expected, which, while not detectable by many of the usual methods, still cause the substances to have different properties from those they possess in vivo. Much is known of the structures of simple substances obtained from biological material, but for the complex bodies, such as the proteins and starches, only the component parts and some of the methods of linking between these have been elucidated. It might appear therefore that the determination of enzyme structure would have to wait for the solution of the problem of the structure of other similar complex bodies. This, however, does not follow necessarily, and, in fact, it appears as if the problem might be reversed. The study of the structure of an enzyme is simplified by the fact that it is possible to follow a change in its structure by the change in activity. This, in a sense, furnishes an additional reagent and makes possible the study of an enzyme in a state more nearly approaching that in which it exists in the living organism. There is therefore a possibility that the determination of the structures of enzymes will precede the determination of the structures of other biological material.

Following this line further, an enzyme may be conceived of as consisting of a molecule showing specific properties, or of a definite grouping in a more complex molecule. This point of view was developed in some detail in Chapter VII, where it was considered that an enzyme grouping was responsible for a given action. In this connection, it is of interest to quote from a paper by Taylor published some years ago:[1] "Upon the basis of the current conception of fermentative acceleration as consisting in the establishment of intermediary reactions, the chemical properties of the different groups within the molecule would determine whether a certain substance could be an accelerator for a certain reaction; and a single molecule could in all correctness be assumed to contain different groups that would qualify it to act as the accelerator for different reactions . . . there is no reason why all the enzymic activities of the pancreatic juice (lipolytic, proteolytic, amylytic, inversion and coagulation) should not be ascribed to the different groups of one organic molecule." The possibilities of isomerism, structural, tautomeric, and stereochemical, are so numerous in a protein molecule, as the latter is conceived of at the present time, that a great number of structures differing only in these ways, could be given, and presumably, given sufficient time and skill, be prepared in the laboratory.

The question of specificity of enzyme actions may be taken up next. These specificities, as stated in an earlier chapter, are striking in many cases, but not unique considered as chemical phenomena. The most obvious reactions in which specificities are used are those included in Qualitative Chemical Analysis (and also Quantitative Analysis). In the reactions involving the identification of the metallic elements, these may be compared to the substrates in enzyme actions, and the reagents used to the enzyme preparations or materials. There are, in both cases, group reagents and individual reagents. With enzymes, for example, amylase, different proteases, emulsin, lipase, etc., act upon certain groups of substrates. Within each group there will be smaller differences for each individual substrate with the group reagent. The conditions must also be kept within certain limits. In qualitative analysis, similarly, hydrogen sulfide might be used as an example of a reagent showing group reactions with certain metallic elements in solution, as well as differences with the individuals in the group, while the conditions of the reaction (such as acidity or alkalinity, etc.) must be kept within certain limits. These analogies might be multiplied indefinitely. One set of phenomena is as remarkable as the other, but familiarity with the one has made these reactions commonplace, while the practical necessity for replacing definite chemical substances by substances as yet not as well characterized and therefore known by names less familiar has resulted in enzyme actions and their specificities acquiring a certain air of mystery. This is unjustified, and their reactions are no more mysterious than are other chemical reactions.

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[1] A. E. Taylor, *J. Biol. Chem.* **5**, 400 (1909).

In the same way the specificities of the oxidizing enzymes may be compared to the actions of the different reagents used in the oxidation of inorganic or organic elements and compounds. The amount of space devoted to these enzymes in this monograph has been small compared with the space devoted to the enzymes accelerating hydrolysis reactions. Fundamentally, this is due to the unsystematic and merely qualitative knowledge of oxidation reactions of organic compounds. The systematization of such reactions on some definite basis appears to be possible at present. The further developments in the study of the oxidizing enzymes will then be based upon these oxidation relations and a satisfactory and reasonable choice of substrate will be possible, instead, as at present, of having the choice depend with each enzyme upon the personal preference of the experimenter.

The lock and key suggestion of Fischer [2] with regard to specificities of enzyme actions may also be taken up briefly. This view was developed from the actions of yeasts and other organisms. He extended it to the enzymes, sucrase and emulsin, acting upon glucosides, and explained the actions by the assumption that the approach of the molecules which is necessary for the bringing about of the chemical process can take place only with similar geometrical structures of the reacting components (enzyme and substrate). To use a picture or model to illustrate the assumption, he suggested that enzyme and glucoside must fit in with one another as lock and key in order for them to exert a chemical action upon each other.

This view and analogy has always aroused great interest. It is a question, however, as to how far the analogy should be taken to hold in connection with elucidating the chemical structures of enzyme molecules. The basic idea of Fischer involves the view of an intermediate or primary addition compound between substrate and enzyme, a view for which various other lines of evidence have been developed. However, the use of the lock and key simile as more than an analogy appears to be open to question. The problem of determining the structures or configurations of chemical molecules which react or combine with each other does not, from the evidence available from other fields of chemistry, depend upon the similarity of the reacting molecules or groupings. In fact, there is just as much, if not more, evidence for chemical reaction occurring with unlike molecules or groups or atoms. The analogy, therefore, while interesting as giving a mechanical picture for certain observations of specificity, can not be used as a means of determining the structure or configuration of the enzyme or active enzyme grouping.

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[2] E. Fischer, *Ber.* 27, 2985 (1894); Cf. E. Fischer and H. Thierfelder, *Ber.* 27, 2036 (1894).

The question of adsorption as a possible explanation for enzyme actions was discussed at some length in the earlier chapters. It may suffice here to state that in the writer's opinion adsorption forms only a step in the mechanism of enzyme actions and is not the dominating factor; that chemical considerations must be used to account for the various steps and combinations (including adsorption) involved, and that, whatever active part adsorption may play is due mainly if not entirely to the chemical properties and reactions taking place with the adsorbing surface and adsorbed substance.

It has been found that various materials occurring in natural sources, sometimes being present together with the enzyme, possess the property of accelerating greatly the enzyme action. For example, bile salts accelerate the action of pancreatic lipase. Such substances are generally known as co-enzymes in enzyme literature. It would appear that any material which accelerates the action of an enzyme preparation may be termed a co-enzyme. Co-enzymes have not been mentioned heretofore in this book. Substances which accelerate enzyme actions have been referred to a number of times. There is apparently no way of deciding where the actions of such substances might be said to end and those of co-enzymes begin. Strictly speaking, they should all be called by the latter name. Although this may fill a psychological need, it seems better not to use a new term for such an action unless it is shown that a new or different chemical phenomenon is involved. In looking about for possibly analogous chemical reactions, it may be pointed out that in certain reactions of inorganic chemistry, it was found that a mixture of two or more substances can accelerate certain changes to a greater extent than any one of them can accelerate these same changes separately. An old well-known reaction, not purely chemical it is true, but more physical in nature if such a distinction may be made, is that involved in the action of the mixed earths in a Welsbach mantle, a mixture of 99% thoria and 1% ceria being found to be most efficient. A recent striking example is that in which it was found possible to oxidize carbon monoxide to carbon dioxide at room temperatures with great rapidity by means of a mixture of various (four) oxides.[3] The number of these examples could be multiplied greatly. The general conclusion seems to be that mixtures of catalysts, or of a catalyst with substances otherwise inert, can be found for certain reactions which produce immeasurably greater increases in the velocities of the reactions than do the single catalysts.

It seems as if co-enzymes should be treated similarly. The nature of the substance termed co-enzyme is known in some cases and may be very simple in character, such as the sodium chloride action with amylase. In other cases it may be quite complex and be chemically unidentified. In view of these relations and the similar reactions in other fields of chemistry, it seems advisable to drop the term co-enzyme from the literature and to attempt to study the problems considered heretofore under that heading from a chemical standpoint.

The study of enzyme actions up to the present time has dealt mainly with problems having as their aim the determination of the chemical structure of enzymes and the careful investigation of the conditions governing the accelerations of the chemical reactions influenced by enzymes. The methods, results, and theories developed in the other branches of chemistry have been used wherever possible. It may be asked now whether the study of enzymes and enzyme actions has aided in the general development of chemical knowledge and theory aside from the specific problems being studied. It may be said that a beginning has been made in this way. Considering the short space of time in which it has been possible, because of the experimental difficulties involved, to carry on accurate enzyme studies, the promise for future usefulness is great.

The industrial uses of enzymes and some analytical work in which enzyme preparations are used as reagents were referred to in Chapter IX. In addition, enzyme studies have aided in clarifying certain general relations of theoretical chemistry. Reference is made to the topic of catalytic reactions primarily, and to the mechanism of chemical reactions in general secondarily. Enzymes have always been considered to be catalysts. The experimental evidence with regard to the mechanism of chemical reactions accelerated by enzymes points unmistakably to the primary formation of compounds between enzyme and substrate, followed by the decomposition into enzyme and products. The possibility of showing this, is due to the nature and properties of the various components involved in the actions. With inorganic, and very frequently with organic, reactions which are catalyzed by added

[3] B. Lamb, W. C. Bray, and J. C. W. Frazer, *J. Ind. Chem. Eng.* 12, 213 (1920).

substances, the evidence for such addition compound formation is not readily obtainable. However, as more careful work is being described in these fields, it is found that more and more reactions show this addition compound formation. The proof is ordinarily more difficult than with enzyme actions because of the properties of the substances and mixtures. As a result of all this work it may be stated that the most probable theory for the mechanism of chemical reactions in which a catalyst takes part involves the formation of an intermediate addition compound with the catalyst. Since a catalytic reaction is defined or described as a chemical reaction in which the chemical composition of one of the products of the reaction is the same as that of one of the initial components, the next step in the chemical theory would include in such a mechanism of chemical reactions all reactions between two or more substances, whether or not the composition of one or more of the substances is unchanged as a result of the reaction. This is the addition theory of chemical reactions. As the material for enzyme actions is presented in this monograph, it would appear as if this theory was assumed initially and that enzyme actions were assigned a place in it. Logically, however, the matter is reversed, and enzyme actions can really be used as a line of evidence in favor of the addition theory. The method of presentation used here is simply a matter of convenience to show the nature of enzyme actions, their relation to chemical reactions in general, and the possibilities for further study.

It is possible that further advances of analogous nature will result from the careful study of enzyme actions. At the present time, reactions of organic chemistry are throwing considerable light on reactions of inorganic chemistry. For example, the mechanism of the apparently simple solution of a metal in an aqueous solution of an acid is being cleared up by the study of the Barbier-Grignard reaction. This is not the place to enter into these questions, but the relations are interesting and promise much for the future.

In concluding this monograph, some of the direct applications of enzymes in living matter may be mentioned, although these relations are still mainly of speculative interest. As far as can be told, the chemical properties of substances in living matter are identical with their properties as obtained in the laboratory. The characteristic of substances in living matter is change, and change in certain definite ways. Enzymes are derived from living matter. They induce changes in definite directions in substances of more or less complex character.

It does not require a far stretch of the imagination to consider enzymes the essential feature of living matter—since living involves chemical changes in certain directions. Also, since enzymes are produced in living matter, the actions appear to be self-perpetuating, in that outside directive agencies are not required to produce the special enzymes needed to bring about the chemical reactions necessary for the continuation of life processes. The interest in enzymes and enzyme actions from the point of view of life processes is therefore justified because of their importance as the directive influences in the chemical reactions of living matter.

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